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COMPETENCE PILUS BIOGENESIS IN *STREPTOCOCCUS PNEUMONIAE*

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Cover: Immunofluorescence microscopy image of *Streptococcus pneumoniae* R6 *hlpA-gfp* (green) expressing competence pili labelled with primary antibody to ComGC and with Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (red)

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COMPETENCE PILUS BIOGENESIS IN *STREPTOCOCCUS PNEUMONIAE*

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To my grandma

ABSTRACT

Horizontal gene transfer drives bacterial evolution and is crucial for the pathogenicity of many bacteria that can exchange genetic material through distinct mechanisms such as natural transformation. *S. pneumoniae,* a common asymptomatic colonizer of the human nasopharynx, is naturally transformable, being able to take up naked DNA and integrate it into its genome. Natural transformation allows pneumococci to acquire new traits, which can provide fitness advantages and contribute to the modulation of carriage and invasive diseases such as pneumonia, meningitis or septicaemia.

Natural transformation is a complex and highly regulated process, which requires bacteria to enter in a distinct metabolic state named competence. During this period, competent pneumococci express all proteins required for transformation. Among them, are the proteins encoded by the *comG* operon (*comGA-G*) implicated in the assembly of pneumococcal competence type IV pili (T4P), which were shown to directly bind DNA. Deciphering how these filaments are assembled is a crucial step in understanding pneumococcal natural transformation and the main focus of this thesis.

Pneumococcal T4P can be several micrometres long and are composed of the major pilin ComGC. We demonstrated that ComGC has intrinsic capacity to polymerize and to assemble into pili. We also solved the high-resolution NMR structure of N-terminal truncated ComGC and found that ComGC has distinct structural features compared to other known type IV pilins and is highly flexible. Based on the structure of ComGC, we further identified a region with potential DNA binding properties, providing the basis to understand the function of competence pili. In addition to our investigation of the major pilin ComGC, we also studied the role of the minor pilins ComGD, ComGE, CopmGF and ComGG in pilus assembly and transformation. We showed that the pneumococcal minor pilins form a minor pilin complex likely to prime pilus assembly. Moreover, we visualized that ComGF is part of the filament and present strong evidence for the role of ComGG as the linker between the minor pilin complex and ComGC.

Pneumococcal colonization and virulence can be influenced by different factors including induction of competence genes and cell morphology. We found that Deterministic Lateral Displacement is a suitable label-free microfluidics technique to sort *S. pneumoniae* into subpopulations based on morphological properties, which can be a relevant tool to understand the mechanisms governing colonization and how bacteria cause disease.

In summary, here we provide a genetic and molecular characterization of the key components involved in pneumococcal pilus assembly and present a novel technique to study *S. pneumoniae* subpopulations.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals throughout this thesis:

I. MUSCHIOL, S.; ERLENDSSON, S.; ASCHTGEN, MS.; **OLIVEIRA, V.**; SCHMIEDER, P.; CASPER DE LICHTENBERG, KT.; BOESEN, T.; AKBEY, U.; HENRIQUES-NORMARK, B., Structure of the competence pilus major pilin ComGC in *Streptococcus pneumoniae*. JBC (2017) 292(34) 14134–14146

II. **OLIVEIRA, V**.; ASCHTGEN, MS.; VAN ARP, A.; TENGSTRÖM, T.; HENRIQUES-NORMARK, B.; MUSCHIOL, S., *Streptococcus pneumoniae* minor pilins are core components of the competence pilus assembly machinery and essential for natural transformation. Manuscript.

III. BEECH, JP.; DANG HO, B.; GARRISS, G.; **OLIVEIRA, V**.; HENRIQUES-NORMARK, B.; TEGENFELDT, JO. Separation of pathogenic bacteria by chain length. Anal. Chim. Acta 1000 (2018) 223e231

CONTENTS

LIST OF ABBREVIATIONS

AOM	Acute otitis media
ARGs	Antibiotic resistance genes
BACTH	Bacterial Adenylate Cyclase Two Hybrid
BSA	Bovine serum albumin
CAP	Community-acquired pneumonia
Cryo-ET	Cryo electron tomography
CSP	Competence stimulating peptide
CU	Chaperone-usher
DC	Critical diameter
DLD	Deterministic lateral displacement
DNA	Deoxyribonucleic acid
dsDNA	Double strand DNA
DUS	DNA uptake sequences
E5	Glutamate at position 5
EPEC	Enteropathogenic *Escherichia coli*
ESBLs	Extended-spectrum β-lactamases
ETEC	Enterotoxigenic *Escherichia coli*
FACS	Fluorescence activated cell sorting
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HGT	Horizontal gene transfer
ICEs	Integrative conjugative elements
iMF	Inertial microfluidic separation
IPD	Invasive pneumococcal disease
KPC	Carbapenemase-resistant *Klebsiella pneumoniae*
LPS	Lipopolysaccharide
MRSA	Methicillin-resistant *Staphylococcus aureus*
NETs	Neutrophil extracellular traps
NMR	Nuclear magnetic resonance
OD	Optical density
PBPs	Penicillin-binding proteins
PBS	Phosphate buffered saline
PCV	Pneumococcal conjugate vaccine

pfu	Plaque-forming unit
PLL-g-PEG	Poly L-lysine-g-polyethylene glycol
pN	Piconewton
PPSV23	Pneumococcal polysaccharide vaccine
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssDNA	Single strand DNA
T2SS	Type II secretion system
T4CP	Type IV coupling protein
T4P	Type IV pili
T4SS	Type IV secretion system
TCP	Toxin-coregulated pili
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infections
UV	Ultraviolet
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization
WT	Wild type

1 INTRODUCTION

1.1 Horizontal gene transfer in bacteria

Bacteria are able to exchange genetic material, a process commonly referred to as lateral or horizontal gene transfer (HGT). Horizontal gene transfer allows bacteria to acquire new genetic material from other bacteria thereby contributing to their genomic diversity and potentially increasing their evolutionary fitness. The exchange of genetic material among bacteria can occur through different mechanisms. In this section, a brief overview of the main mechanisms including conjugation, transduction and transformation and their impact will be presented.

1.1.1 Conjugation

Conjugation is the mechanism whereby a donor bacterium exchanges genetic material with a recipient bacterium. The process is well-conserved among Gram-negative and Gram-positive bacteria as well as archaea (1). The genetic material transferred during conjugation can either be conjugative plasmids or integrative conjugative elements (ICEs). The major difference between them is that, once transferred to the recipient bacterium, conjugative plasmids remain as extra chromosomal elements and replicate independently, whereas ICEs need to be integrated into the bacterial genome to be able to propagate during cell division (2).

Conjugation requires direct cell-to-cell contact between donor and recipient bacteria through the formation of a conjugation pilus. The assembly of conjugative pili requires a sophisticated type IV secretion system (T4SS) also known as transferosome (3). The pilus is assembled by the donor bacterium and establishes the interaction with the recipient bacterium. This donor-recipient interaction is also called mating pair formation and it is crucial for DNA translocation. The exact mechanism through which the mating pair is formed remains elusive. In Gram-negative bacteria, interactions between an adhesin present on the pilus tip of the donor bacterium, with the lipopolysaccharides (LPS) on the surface of recipient bacteria have been reported (4). However, another study showed that mutations in the LPS synthesis pathway had only a modest impact on conjugation (5). Once the mating pair is formed, the pilus retracts, bringing donor and recipient bacteria in close proximity. Then, a signal is transmitted to the transferosome indicating that DNA processing and transfer should be initiated (6). This signal is produced by the recipient bacterium. It can either be peptides acting as pheromones or signal peptides secreted in a cell density-dependent manner and regulated by quorum-sensing (2, 7). The signal is then sensed by the donor bacterium and transferred along the transferosome to another complex named relaxosome. The relaxosome is formed by a conjugative DNA-processing enzyme

(relaxase) and accessory proteins bound to a specific DNA sequence called origin of transfer (*oriT*) (8). Relaxases create site- and strand specific nicks within *oriT* thereby processing and preparing the transfer of single-strand DNA. The interaction between the transferosome and the relaxosome is established with the help of a protein named type IV coupling protein (T4CP) (9, 10). T4CP is a crucial intermediate, which responds to the DNA transfer activation signals, and it mediates the relaxase-DNA complex translocation through the transferosome into the recipient bacterium. Finally, once in the recipient cell, the incoming DNA is recircularized by the relaxase and the complementary strand is synthesized to generate stable transconjugants (2, 11).

Conjugation has been extensively studied in the F-plasmid transfer system in *Escherichia coli* and in F-like plasmid systems in association with the spread of antibiotic resistance genes (12). Inhibiting conjugation is therefore an attractive approach to tackle antimicrobial resistance and the spread of diseases. However, conjugation is not only restricted to bacterial cells as donor and recipient, as the plant pathogen *Agrobacterium tumefaciens* has the ability to use conjugation to transfer oncogenic DNA into plant cells to cause crown gall disease (13).

1.1.2 Transduction

Transduction refers to the exchange of DNA carried out with the help of bacteriophages (or phages), transferring DNA from one bacterium to another. Bacteriophages are viruses that infect and replicate only in bacteria. These viral particles are extremely diverse in size, morphology, and in genomic organization, but typically consist of genomic material enclosed in a phage-encoded capsid (head), which protects and mediates its delivery into the next host bacterial cell (14). In lysogenic bacteria, phages are integrated into the host bacterial genome as prophages where they silently replicate during cell division (15). Then, either spontaneously or in response to external factors such as, DNA-damaging agents or antibiotics, prophages can excise and enter into the lytic life cycle to form mature phage particles (16). The first step of the lytic cycle involves the circularization of the viral genome. Subsequently, the viral genome undergoes several replication cycles to generate new phage components and to form long DNA concatemers, which are packaged into the phage head (11, 15). The packaging is mediated by a phage terminase enzyme, which recognizes a phage-specific packaging site and cleaves the DNA creating a free end to which the enzyme remains bound. The DNA-terminase complex is directed to the phage head to initiate DNA packaging. Once the packaging is completed, the terminase makes another cut in the DNA to dissociate it from the phage head. The enzyme remains bound to the concatemer end and begins a new round of DNA packaging into another empty capsid. Finally, mature phage particles are able to lyse the host and infect new bacterial cells (17).

Occasionally, some parts of the host bacterial genome can also be transferred during transduction, either by generalized or specialized transduction. In generalized transduction, the phage packages and transfers any portion of bacterial DNA. This happens due to mistakes by the terminase, which wrongly recognizes packaging sites in the host bacterial genome and mediates its packaging (15). This mechanism was originally discovered in *Salmonella* phage P22 and together with phage P1 of *E. coli;* they represent two well-studied models of generalized transduction (18, 19). In contrast, during specialized transduction only a specific set of genes is transferred. Studies of the *E. coli* λ phage have shown that the abnormal prophage excision brings adjacent segments of the host bacterial DNA together. The lytic cycle proceeds naturally resulting in a hybrid molecule which is recognized by the terminase, packaged and transferred to new bacterial cells (20).

Recently, a third type of transduction, named lateral transduction, has been identified in *Staphylococcus aureus* phages (21). This transduction mechanism also involves the transfer of bacterial chromosomal DNA but unlike the aforementioned mechanisms, this process appears to be programmed and not the result of an erroneous process. Importantly, staphylococcal prophages do not follow a typical lytic cycle as they excise late in their life cycle. As a consequence, the terminase initiates DNA packaging in situ from integrated prophages. This results in the transfer of large spans of bacterial chromosomal DNA adjacent to prophages at much higher frequencies than genes located elsewhere (21). Thus, it has been reasoned that transduction is not only a major mechanism to spread virulence factors, but also an evolutionary adaptation to ensure phage survival and to increase its host´s fitness (22).

1.1.3 Natural Transformation

Natural transformation refers to the process by which free DNA from the environment can be actively taken up by bacteria. The extracellular DNA used as substrate for transformation can be secreted into the environment by other bacteria or released by dying bacteria. Notably, extracellular DNA is also a major component of biofilms formed by many pathogenic bacteria (23, 24). The stability of the extracellular DNA in the environment will dictate the bacterial transformation efficiency.

To date, about 80 bacterial species have been identified that can undergo natural transformation, including representatives of both Gram-negative and Gram-positive bacteria as well as cyanobacteria and archaea (25). All transformable bacteria are thought to share common, conserved mechanisms to assemble surface located structures called pili that are required for DNA uptake. The only known exception is *Helicobacter pylori* which uses a conjugation-like system for DNA transport (26).

Natural transformation is exclusively mediated by the recipient bacteria and all required proteins are encoded in the core genome (25). Transformation is a stepwise, energy-requiring process and involves the following stages: (i) binding of double-strand DNA to the bacterial cell, (ii) processing of DNA and transport into the cytoplasm and (iii) recombination and integration of the transforming DNA into the bacterial genome (27). Thus, natural transformation requires a coordinated expression of multiple proteins to ensure efficient DNA uptake. This is achieved through a highly regulated process during which bacteria first need to become competent. During this distinct physiological state, all the proteins required for DNA uptake, processing and recombination are produced. Depending on the species, the competence window varies but is typically time-limited in response to distinct environmental cues such as cell density, nutrient availability or stress conditions. For instance, in *Haemophilus influenzae*, competence is induced *in vitro* in response to a nutrient downshift or signals of starvation (28). In other bacteria, like *Legionella pneumophila,* competence is induced in response to DNA damaging agents or UV radiation (29). The formation of biofilms also seems to stimulate the rate of transformation in some human pathogenic bacteria such as *Campylobacter jejuni* and *Streptococcus mutans* (30, 31). In addition, some bacteria synchronize their competence state with other physiological responses including growth arrest or bacterial competition. In *Bacillus subtilis,* competence is induced at high cell density or in the beginning of the stationary phase and only 10-20% of the bacteria in the culture are becoming competent (32). In addition, competence induction in *B. subtilis* leads to cell growth arrest which can last several hours once cultures are diluted into fresh medium (33). Bacteria in this bistable state are also more tolerant to different antibiotics like penicillin, kanamycin and oxolinic acid, a quinolone-type antibiotic (33, 34). In nature, it is plausible that this long cell growth arrest could function as a mechanism to ensure proper chromosome maintenance after transformation and to survive in complex environments like soil (27). Among other transformable bacteria, *S. pneumoniae* was also shown to undergo growth arrest upon induction of competence *in vitro*. This was proposed to help pneumococci to maintain their genomic integrity during transformation without compromising cell viability (35). In contrast to *B. subtilis*, competence in *S. pneumoniae* is induced early during exponential growth and, in laboratory strains including D39, more than 95% of the culture can become naturally competent (36).

Different theories regarding the costs and benefits of natural competence for transformation have been proposed including that competence functions as repair mechanism or as an adaptation strategy to new environments through the acquisition of new traits (37). These hypotheses are not mutually exclusive and the reason to become competent is likely bacteria-dependent in response to the challenges of the surrounding environment.

1.1.4 HGT and the spread of antibiotic resistance

Antibiotic resistance is an emerging major global health threat as antibiotics are losing effectiveness against an increasing number of bacterial pathogens. It is estimated that worldwide at least 700 000 people die annually due to drug-resistant diseases (38). With the burden of antibiotic resistance increasing globally, it was predicted that this number will increase up to 10 million deaths annually by the year 2050 (39).

Resistant bacteria can rise and spread through selective pressure created by antibiotics favouring resistance or alternatively, antibiotics eliminate susceptible bacteria allowing resistant bacteria to propagate (40). They often represent a major concern in healthcare centres and hospital settings and their rise in community-acquired infections is alarming (41). HGT plays a central role in the rapid spread of resistance since bacteria easily exchange genetic material and transfer antibiotic resistance genes (ARGs) to multiple related or unrelated species leading to serious infections and outbreaks (40, 42). Considering the three main mechanisms of HGT, conjugation is often viewed as the main mechanism responsible for the spread of antibiotic resistance associated with hospital-acquired infections. The reason for that is related to the high occurrence of plasmids carrying ARGs that can be promiscuously transferred to a wide range of recipient bacteria (42-44).

Plasmid-mediated resistance to β-lactam antibiotics exemplifies how ARGs have been disseminated by intra- and interspecies conjugation (45, 46). Resistance to β-lactam antibiotics can be acquired by the action of extended-spectrum β-lactamases (ESBLs) and carbapenemases which hydrolyse β-lactam antibiotics like penicillin and carbapenem (47). The development of carbapenem resistance has become a public health threat since carbapenems are among the antimicrobials considered as last-line antibiotics. This type of antimicrobial resistance has been observed in clinically relevant carbapenememase-producing *Enterobacteriaceae* such as *E. coli, Klebsiella* spp., and *Enterobacter* spp. (48). In 1996, carbapenemase-resistant *K. pneumoniae* (KPC) were first reported in the USA and a number of plasmids carrying KPCs have been described that can be transferred by HGT (45, 49-51). The alarming rate of carbapenem resistance spreading is of major concern and has resulted in outbreaks worldwide. In Europe, its prevalence is high mainly in southern and eastern countries whereas it still remains low in northern ones (52).

Another example where HGT, namely conjugation, is implicated in the spread of antibiotic resistance and virulence determinants is the pathogen *S. aureus,* which is a major cause of nosocomial and community-associated infections (53). The treatment of infections caused by this pathogen is being challenged by the development of resistance to β-lactam antibiotics through the acquisition of the *mec* gene, which encodes a low-affinity penicillin-binding protein and confers

resistance to methicillin and most β-lactam antibiotics (54). The methicillin resistance gene is often plasmid-encoded, hence can be easily transferred by HGT. As a consequence, methicillin-resistant *S. aureus* (MRSA) has become a major healthcare burden worldwide. The prevalence of MRSA infections in Europe is somewhat similar to what has been described for carbapenem resistance above, with southern countries having a higher prevalence compared to northern countries (55). Vancomycin is frequently used to treat MRSA infections and although still sporadically, clinical isolates of vancomycin-resistant *S. aureus* (VRSA) have been identified (56). VRSA has evolved from MRSA by acquisition of a plasmid containing vancomycin resistance genes from *Enterococcus faecalis* likely by conjugation (57). Taken together, these examples show that conjugation plays a major role in the occurrence and spread of antibiotic resistance, however, the impact of transduction and natural transformation should not be disregarded.

Phages are often pointed out as an alternative approach to antibiotics in the fight of multi-drug resistance, and phage therapy has been successfully used to treat infections caused by many pathogens including *S. aureus, E. coli* and *Streptococcus* spp. (58, 59) However, it should not be ignored that many of these pathogens carry phages that could potentially transfer genetic material. Although a direct evidence that transduction can occur in clinical settings is currently lacking, *in vitro* transduction of phages in *Enterococcus, Pseudomonas* and *Staphylococcus* spp has been observed (42). In laboratory conditions, the majority of bacterial species tested presented relatively low transduction frequencies of phages carrying a particular ARG (10^{-5}-10^{-9} transductants/plaque-forming unit (pfu)). However, in *S. aureus* a phage carrying a streptomycin resistance gene was able to transduce at a much higher frequency (10^{-1} transductants/pfu), suggesting that these phages could contribute to the spread of antibiotic resistance (60).

The limited number of known natural transformable bacteria identified to date may underestimate the importance of transformation in the development and spread of antibiotic resistance. According to the World Health Organization (WHO) priority list of antibiotic resistant bacteria, which includes 12 bacterial species, 8 of those are known to be naturally transformable (42). For example, in multi-drug resistant *Neisseria gonorrhoeae,* which are increasingly more difficult to treat, it is believed that the majority of gonococcal ARGs have been acquired by natural transformation (61). Interestingly, many of the species present on the WHO list, such as *A. baumannii* and *P. aeruginosa*, live in communities and can form biofilms that contain extracellular DNA as their major component.

In summary, conjugation is considered as the primary mechanism responsible for antibiotic resistance. However, the impact of transduction and natural transformation cannot be underestimated.

1.2 Bacterial pili: an overview

Bacteria express distinct non-flagellar, filamentous structures termed pili or fimbriae protruding from their surfaces. Bacterial pili were first discovered in Gram-negative bacteria in the late 1940s and since then they have been extensively studied in association to different diseases (62, 63). Two decades later, pili were also identified in Gram-positive bacteria, and today it is also known that pili are widely present among these bacteria (64-66). Different types of pili can be distinguished based on their morphology and assembly pathways, including chaperone-usher pili, curli, type IV pili, conjugative pili, type V pili and sortase-mediated pili (Figure 1 and Figure 2). They can display a wide range of functions, such as adhesion to host cells, biofilm formation, twitching motility, and exchange of genetic material, being important for virulence of many pathogenic bacteria (63, 65). In this section, a summary of the structure, function and assembly mechanism of different pilus systems will be presented.

Chaperone-usher (CU) pili are ubiquitously present on the surface of many Gram-negative pathogenic bacteria (67). CU pili are multi-subunit polymers non-covalently linked that require two accessory proteins for their assembly in the outer membrane: a periplasmic chaperone protein and an outer-membrane pore-forming protein named the usher. The chaperone facilitates the correct folding of pilin proteins and presents them to the usher. At the usher, pilins are assembled and the growing pilus is translocated to the bacterial surface through the usher pore (68). CU pili can be up to 3 µm long and in many species, they are composed of thousands of pilus subunits forming a thick helical rod connected to a thin fibrillar tip (fibrillum) formed by distinct subunits. However, this architecture is not conserved in all CU pili as some lack the tip fibrillum while others lack the rod structure (68).

CU pili have been extensively studied in the context of urinary tract infections (UTI) caused by uropathogenic *E. coli* (UPEC). These bacteria assemble two types of CU pili, type 1 and P pili, which will be used here as CU pili models. Components of type I and P pili are encoded in the *fim* and *pap* operons, respectively (69). Both type 1 and P pili form a short, thin tip fibrillum assembled on a thicker helical rod, 1-3 µm in length. They differ in the number of pilus components, which form the tip fibrillum. In type I pili, the tip fibrillum consists of one copy of FimG and FimF pilus subunits. FimF connects the fibrillar tip to the pilus rod which is composed of more than thousand copies of the pilus subunit (FimA). P pili present a similar architecture but have longer tip fibrillae. The fibrillar tip is formed by an individual copy of PapF together with 5-10 copies of PapE. In addition, one copy of the PapK subunit links the tip fibrillum to the rod. The rod is composed of multiple copies of the main subunit (PapA) (63, 69). At the distal end of the fibrillar tip, both type 1 and P pili present an adhesin protein (FimH and PapG,

respectively), which interacts with distinct host-cell receptors. FimH interacts with D-mannosylated receptors on bladder epithelial cells, whereas PapG binds to galabiose (Galα1-4Gal)-containing glycosphingolipids present on the kidney epithelium (70, 71). Thus, type 1 pili are involved in the adhesion to the bladder, whereas P pili target the kidney. Together, they play an important role in the development of UTIs allowing the bacteria to translocate from the bladder to the upper urinary tract (68, 72). For this reason, CU pili have been considered attractive antibacterial targets to prevent UTIs. Mannosides, mannose derivatives, which compete with the cognate receptor of the FimH adhesin, have been developed and shown promising results in treating chronic UTIs in murine models (73, 74).

Curli are coiled fibres assembled by many enteric bacteria. They are involved in different functions including host cell adhesion, invasion and biofilm formation (75). Curli can be up to several micrometres long and resemble amyloid fibres. Historically, amyloid fibres are formed by unfolded or misfolded proteins that are prone to aggregate and represent pathological hallmarks of some neurodegenerative diseases such as Alzheimer's and Parkinson's (76). However, amyloid fibre formation can also be the result of a functional biogenesis system comprising properly folded proteins. Curli are an example of a such system, which is why they are considered as functional amyloid fibres (75).

Curli formation is well-studied in *E. coli* and *Salmonella* spp. (77). In *E. coli*, proteins involved in curli formation are encoded in the *csgBAC* and *csgDEFG* operons (78). Curli are composed of two components non-covalently linked: the major structural subunit CsgA (also named curlin) and the pilus assembly nucleator CsgB. Curli assembly must be highly controlled to avoid the toxicity found in disease-associated amyloid fibres and incorrect curli assembly could lead to the disruption of bacterial membrane integrity (79). CsgD is the main regulator that coordinates curli biogenesis. To protect bacteria from the toxicity associated with an accidental periplasmic accumulation of curli subunits, the periplasmic protein CsgC interacts with CsgA to prevent premature fibre formation (80). Two accessory proteins named CsgE and CsgF mediate the secretion and assembly of curli trough the transport channel formed by CsgG (81).

Curli are resistant to proteolytic degradation and distinct physiological conditions including osmolarity and pH variations. In *E. coli,* it was shown that CsgA subunits fibrillate *in vitro* over a wide range of pH values and salt concentrations (79). The ability of CsgA to form fibrils in a large number of conditions may facilitate the adaptation of *E. coli* to different environments.

The crosstalk between bacteria can also mediate curli assembly. Evidence of such collaboration has been shown *in vitro* by growing an *E. coli* strain lacking *csgB* but expressing *csgA* in close proximity to another *E. coli* strain encoding *csgB* but

lacking *csgA*. Interestingly, the soluble CsgA subunits polymerized and formed functional curli on the surface of the strain only expressing CsgB (81), a process known as interbacterial complementation. Interbacterial complementation has also been observed in multispecies biofilms formed by *E. coli* and *S. typhimurium*. These bacteria can share curli subunits between them to assemble functional fibres thereby restoring bacterial adhesion in multispecies biofilm communities (82). In nature, *E. coli and Salmonella* spp. often coexist in the same niche, thus it is plausible that they collaborate mutually to adhere better and form more resistant biofilms providing fitness advantages and better survival options.

Type IV pili (T4P) are filaments which can be several micrometres in length. They are widespread among Gram-negative and Gram-positive bacteria as well as in archaea (63, 65). In the latter, these filaments are named the archaeal flagellum or 'archaellum' (83). T4P have been implicated in several functions including adhesion, cell aggregation and biofilm formation, bacterial motility, DNA uptake and electron transfer (65). A comprehensive description of the major T4P components, their assembly and functions will be presented in detail later.

Conjugative pili are commonly found in both Gram-negative and Gram-positive bacteria (84). These tube-like filaments play a crucial role in bacterial conjugation by mediating cell-cell contact and the exchange of genetic material. Conjugative pili can be several micrometres long and their assembly, extension and retraction requires multiprotein nanomachines called type IV secretion systems (3, 85). Two well-known T4SSs are the F plasmid transfer system in *E. coli* and the Ti plasmid injection system in plant pathogen *A. tumefaciens* (86). The nomenclature from *A. tumefaciens* is often used to describe these systems and it will be used herein. The components of the Ti plasmid injection system are encoded in two operons, *virB* and *virD*. The *virB* operon encodes 11 structural components (VirB1-VirB11) and the *virD* operon encodes four regulatory and accessory proteins (VirD1-VirD4) (87). The T4SS in *A. tumefaciens* is formed by a transmembrane secretion complex composed of three ATPases (VirB4, VirD4 and VirB11), structural components (VirB3, VirB6 and VirB8 that form a complex in the inner membrane and VirB7, VirB9 and VirB10 that form a complex in the outer membrane) and the pilus made of the major pilin subunit VirB2 and a minor subunit (VirB5). In addition, a hydrolase (VirB1) breaks down the peptidoglycan layer thereby facilitating T4SS formation (87). *A. tumefaciens* uses its conjugative pili to inject DNA into plant cells, which leads to the formation of plant tumours (13).

Significant progress in deciphering the structural organization of T4SSs has emerged from studies of the F plasmid transfer system encoded by the *tra* operon in *E. coli*. Cryo-electron microscopy studies of two members of the F pilus family originated in an atomic model where the major pilus subunit TraA is polymerized in a five-

start helical filament (88). Interestingly, the authors also found that pilin subunits form a stoichiometric protein-phospholipid unit in a 1:1 ratio (88). It was therefore proposed that the incorporation of phospholipids into the filament helps the exchange of DNA during conjugation by making the pilus lumen moderately electronegative. It was further suggested that the presence of phospholipids reduces the energetic barrier for pilus assembly enabling an efficient cargo delivery into the recipient bacterium (88). Recently, unprecedented F pilus-associated structures in the native bacterial cell envelope were identified by in situ cryo-electron tomography (ET) (89). Four distinct structures were identified: the T4SS composed of the outer and inner membrane complexes connected by a central channel with (i) or without (ii) pilus visible at the outer membrane. In addition, two novel structures were visualized, (iii) one containing the F-pilus attached only to a thin capped stalk spanning the periplasm and (iv) one where the F-pilus was associated with the outer membrane without any visible periplasmic structures associated. Based on these results, it was then proposed that the structures (i) and (ii) were involved in conjugation and plasmid transfer while the two novel structures (iii) and (iv) are likely to facilitate bacterial aggregation. Altogether, is believed that coordinated assembly of these different structures would increase the probability of plasmid transfer (89).

Type V pili were first characterized in 2016 in the human oral pathogen *Porphyromonas gingivalis*, the causative agent of severe adult periodontitis and gingivitis (90). They are formed by a divergent set of proteins found particularly in the class Bacteroidia, which is why these pili are also known as Bacteroidia pili (90). Bacteroidia pili play an important role in virulence contributing to bacterial adhesion and biofilm formation (91, 92). *P. gingivalis* assembles two morphologically distinct type V pili: long (0.3-1.6 µm) or short (80-120 nm) pili (93, 94). Both pili are encoded in similar operons by genes coding for the structural pilin that forms the pilus stalk, anchoring pilins which connect the pilus to the bacterial membrane, minor subunits including the tip pilin and additional regulatory proteins (95, 96). The current understanding is that these pili are synthesized using a lipoprotein-proteinase pathway (90). Pilus subunits are translocated as lipoprotein precursors to the periplasmic side of the inner membrane where the precursors are lipidated and are cleaved by a signal peptidase. Then, modified pilins are transported across the outer membrane through an unknown pore with the help of a chaperone-like lipoprotein, which likely recognizes the lipid moiety in the pilus subunit. After secretion, stalk pilin subunits, and tip pilins, but not anchor pilins, undergo a second proteolytic cleavage to remove the lipidated fragment prior to pilus assembly. Further studies are required to understand the exact pilus assembly mechanism. Interestingly, the use of extracellular peptides that target the major pilus subunit leads to disruption of pilus polymerization and hence reduce the ability of *P. gingivalis* to form biofilms, indicating a promising strategy for treatment and/or prevention of periodontal diseases (91).

Sortase-mediated pili are exclusively found in Gram-positive bacteria. These bacteria lack the outer-membrane present in Gram-negative bacteria, which is why they require a distinct mechanism for pilus assembly and attachment to the bacterial surface. The assembly of sortase-mediated pili relies on the transpeptidation activity of enzymes named sortases (97). Sortases are cell surface-associated or anchored transpeptidases, which recognize a conserved C-terminal LPXTG motif in the substrates and mediate their attachment to the peptidoglycan or covalently link pilin proteins to assemble pili. Among the six sortase classes (A, B, C, D, E, and F), class C sortases are responsible to covalently link pilus subunits into polymers whereas others have cell wall anchoring functions (98).

Sortase-mediated pili were first identified in 1968 in the animal pathogen *Corynebacterium renale* (64). Later, it was shown that these pili are present in many other Gram-positive bacteria including *Streptococcus* and *Enterococcus* spp., where they play an important role in host-cell adhesion and biofilm formation (97). Two types of sortase-mediated pili have been described: short and thin structures which can be up to 500 nm in length and longer flexible pili which can be up to 3 µm long (99). A typical sortase-mediated pilus is composed of three building blocks: the basal pilin responsible for anchoring of the pilus to the peptidogylcan, the backbone pilin and the tip pilin with adhesive properties. These pilus components are encoded in genes clustered together with genes encoding pilus-specific sortases (97). Pilus-specific sortases initiate pilus assembly by cleaving the LPxTG motif of the tip pilin, which is then covalently linked to the backbone pilin. The backbone pilin is also recognized and cleaved by a dedicated sortase, which catalyses the linkage between backbone pilins promoting pilus polymerization. The polymerization is completed when the basal pilin is added to the pilus, which together with a dedicated sortase functions as the termination signal. The pilus backbone is then covalently linked to the basal pilin, which anchors the entire structure to the peptidoglycan. The adhesive properties of these filaments are important in the pathogenesis of several *Streptococcus* species. For this reason, pili have been attractive targets for vaccine development to combat infections caused by these Gram-positive pathogens. For instance, the crystal structure of the backbone pilin in Group B *Streptococcus* revealed that most of the protective epitopes are localized in a single domain. This information could help in the development of a pilus-based vaccine against Group B streptococcal infections which still represent a serious threat to new-borns (100, 101).

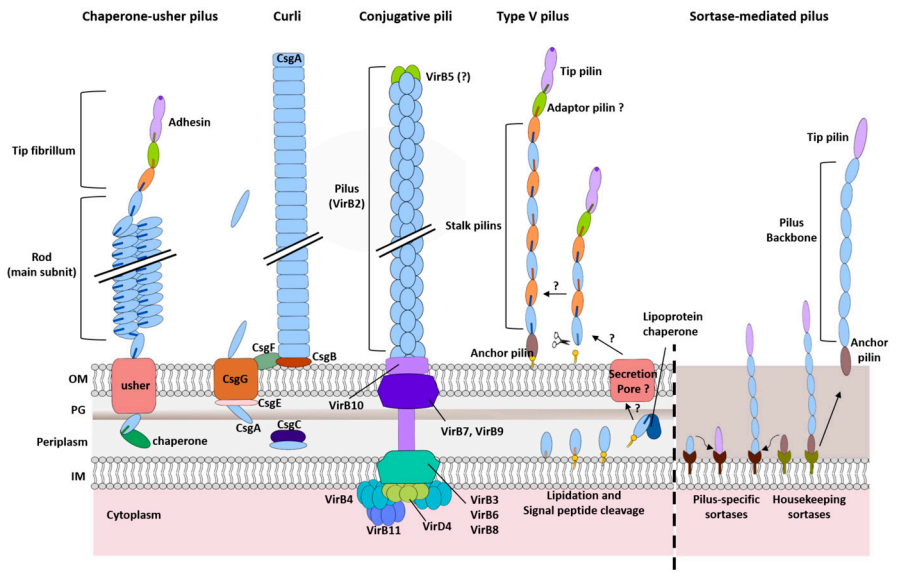

Figure 1. General organization of chaperone-usher pili, curli, conjugative pili and type V pili in Gram-negative bacteria and sortase-mediated pili in Gram-positive bacteria. OM – outer membrane, PG – peptidoglycan, IM – inner membrane. Question mark (?) represents unknown proteins or steps during pilus formation.

Taken together, pili are widespread bacterial surface structures that represent crucial virulence factors for many pathogenic bacteria. Research in the field of microbial pathogenesis has particularly focused on understanding the different type of pili and their associated functions. Interestingly, different types of pili share the same pilus associated function, such as adhesion or biofilm formation, which represent a common strategy used by different pathogens to colonize and cause disease in various niches. Moreover, recent structural investigations of these bacterial systems have unquestionably provided additional valuable information. Thus, the continuous development of higher-resolution techniques will be of great importance to further understand the complexity of these structures, which will help to develop novel antimicrobial strategies that target key pilus components.

1.3 Type IV pili: a closer look

T4P were initially described in *P. aeruginosa* and subsequently identified in many other Gram-negative human pathogens including *Neisseria* spp. (102). Due to their architectural characteristics, that involve crossing both the inner and outer bacterial membranes, they were initially thought to be present only in Gram-negative bacteria. However, later reports also demonstrated their existence in

Gram-positive bacteria as well as in archaea (65). T4P can be several microns in length and have been observed as individual structures or in bundles depending on the species (65, 103). These structures are also remarkably flexible being able to bear stress forces greater than 100 pN (104). T4P are predominantly composed of numerous copies of a single protein called major pilin. In addition, minor pilin proteins, which are present at lower abundance, can also be incorporated into the filament (65, 105).

1.3.1 Type IV pili biogenesis: key components

T4P assembly requires sophisticated machineries composed of distinct multiprotein complexes which can involve up to 15 or more proteins depending on the species (106). In Gram-negative bacteria, the T4P machinery spans both inner and outer membrane and shares homology with the type 2 secretion system (T2SS), which is involved in the translocation of proteins from the periplasm to the outer membrane (103). Recent advances in genomic sequencing and structural biology started to provide a better understanding of T4P systems also in Gram-positive bacteria and revealed that they share remarkable similarities with T4P systems in Gram-negative bacteria. T4P biogenesis has been extensively studied in Gram-negative bacteria, where T4P were first identified. In this section, a general overview of the main components involved in pilus assembly with focus on T4P in Gram-negative bacteria will be presented.

1.3.1.1 Pilins

Pilins, in particular the major pilin, are the primary components of T4P. In addition, minor pilins present in lower abundance can also be part of the filament (107). Pilin proteins are synthesized as precursors referred to as prepilins. Prepilins share a hydrophilic leader peptide (signal peptide) typically ending with a glycine residue, which is recognized and cleaved by a unique leader peptidase (prepilin peptidase). All pilin proteins also share a conserved hydrophobic N-terminal domain, which allows them to be anchored in the inner membrane, with the signal peptide facing the cytoplasm and the C-terminal domain facing the periplasm. The C-terminal domain is highly variable among pilins, and it is associated with the functional diversity of T4P (108).

Conventionally, T4P pilins can be divided into two classes, type IVa and IVb, based on the length of their N-terminal signal peptide. Type IVa pilins possess short signal peptides (with less than 10 residues) and result in the assembly of thinner filaments, whereas type IVb pilins have longer signal peptides that can range from 15 to 30 residues. The first residue of the mature protein is typically a phenylalanine for type IVa pilins and another hydrophobic residue for type IVb pilins (e.g. methionine, leucine or valine). Type IVa prepilins exhibit considerable

sequence homology in their N-terminal hydrophobic domain when compared to their type IVb counterparts (106, 107). T4P composed of type IVa pilins can be found in plant, animal and human pathogens including *Pseudomonas, Neisseria*, and *Dichelobacter* as well as in environmental genera such as *Thermus, Myxococcus* and *Deinococcus*. Pili formed by type IVb pilins are more diverse and have been divided in different sub-families. Pilins belonging to the class of type IVb have been characterized in enteric bacteria such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), *S. enterica* serovar Typhi and *Vibrio cholerae* (107).

1.3.1.2 Prepilin peptidase

A crucial step of T4P assembly is the cleavage and processing of prepilins into mature pilins prior to their oligomerization into pili. This requires the proteolytic activity of a family of dedicated proteases called type IV prepilin peptidases. The first prepilin peptidase was described in *P. aeruginosa* and is encoded by the gene *pilD* (109). Later, PilD-like prepilin peptidases have been identified in both Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, PilD is located on the cytoplasmic side of the inner membrane (110). Prepilin peptidases are typically composed of two domains: one peptidase domain which removes the hydrophilic signal peptide present in type IV prepilins and a second domain responsible for N-methylation of the first residue of mature pilins (111). Prepilin peptidases recognize and cleave after a conserved glycine or alanine which is followed by a stretch of primarily hydrophobic residues present in the signal peptide (109). Cleavage of the signal peptide, but not pilin methylation, is crucial for the assembly of functional pili (112-114).

1.3.1.3 Assembly/retraction ATPases and platform protein

Besides major and minor pilin proteins, all T4P systems also encode a conserved integral membrane protein and assembly and retraction ATPases. The integral membrane protein is believed to function as an assembly platform where pilus assembly takes place. In *P. aeruginosa*, the assembly platform was shown to interact with the assembly ATPase via its N-terminal cytoplasmic domain (115). The action of cytoplasmic ATPases is required to provide the energy to power pilus assembly and retraction. When these enzymes bind and hydrolyse ATP, conformational changes in the ATPase are induced, which will drive pilus polymerization (assembly ATPase) or depolymerization (retraction ATPase). The crystal structures of different ATPases revealed that these enzymes form hexameric rings (116-118). How exactly ATP is used to drive pilus polymerization is still not clearly understood. However, two main models have been proposed: (i) a rotation model and (ii) a compression model. The rotation model is based on the structure of the assembly ATPase present in *Geobacter metallireducens* and cryo-ET imaging of the T4P machinery of *M. xanthus* (119, 120). In this model, ATP hydrolysis

leads to conformational changes in the assembly ATPase, which are transferred to the platform protein and induces its rotation. This movement forces the pilus subunits out of the membrane and opens a gap around the base of the filament that will allow the docking of the next incoming pilus subunit (118-120). In *G. metallireducens* it was suggested that the cytoplasmic domains of the platform protein are inserted into the lumen of the assembly ATPase and the transmembrane domains are located underneath the growing fibre. Alternatively, cryo-ET analysis of the *M. xanthus* T4P machine proposed that the cytoplasmic domains of the platform protein are not part of the lumen of the assembly ATPase but instead associated with the top (120). In both cases, the resulting energy from ATP hydrolysis is transferred to the platform protein causing its rotation to promote assembly of the pilin subunits into T4P (119).

The compression model proposes that the platform protein forms a ring and functions as a gate that opens and closes to control pilin assembly or disassembly. In this model, ATP binding and hydrolysis leads to closing of the gate and in order to release the compression force generated, subunits of the growing filament are pushed outwards allowing the platform protein to return to its relaxed state for the docking of the next subunit (121). Thus, this model does not consider a rotation of the platform protein but only conformational changes in the protein itself (121). Conceptually, it represents a simpler model and further studies are needed to support and validate this model.

In both rotation and compression model, the retraction ATPase activity results in opposing motions that will drive the pilus depolymerization back into the membrane (118-121). While assembly ATPases are intrinsically present in T4P, only some T4P systems employ retraction ATPases that lead to pilus depolymerization. For instance, retraction ATPases have been well documented in *P. aeruginosa* and *Neisseria* spp. in which the major retraction motor is PilT. Interestingly, both systems possess additional retraction ATPases, one (PilU) in *P. aeruginosa* and two (PilU and PilT2) in *Neisseria spp.* (122, 123), which complement the function of PilT. In both, *Neisseria* and *Pseudomonas* deletion of the main retraction ATPase results in loss of pilus associated functions, such as DNA uptake or twitching motility (124-126). However, in *N. gonorrhoeae* retraction was still observed in the absence of all three retraction motors, although at much lower speed, (127). These observations suggest that gonococcal T4P retraction can occur spontaneously, by the action of an unknown protein, or alternatively, by the antagonistic action of the assembly ATPase. In fact, *Caulobacter crescentus* tad T4P lack a retraction ATPase but possess a bifunctional ATPase that can power both pilus elongation and retraction (128). Similarly, *V. cholerae* toxin-coregulated pili (TCP) are retractile despite lacking a retraction ATPase, and pilus retraction was proposed to be mediated by a single minor pilin (129).

1.3.1.4 Secretin subcomplex

In Gram-negative bacteria, pilus assembly occurs in the inner membrane. To reach the bacterial surface the filament needs to pass through the outer membrane. This is accomplished by the multimeric protein called secretin. The secretin forms a pore in the outer membrane through which the pilus can pass (130, 131). In many T4P encoding bacteria this pore is formed by the protein PilQ, which is well conserved among T4P systems in Gram-negative bacteria (121). Cryo-ET studies in *T. thermophilus* revealed that PilQ is composed of a wide periplasmic vestibule with one gate on each side, which are closed in the absence of a pilus to maintain the integrity of the outer membrane (132). Thus, the growing pilus will trigger conformational changes in the secretin to open the gates and to allow the filament to cross the outer membrane. The proper assembly and placement of the secretin in the outer membrane is often assisted by an outer membrane lipoprotein called pilotin (133). In *N. gonorrhoeae* and *M. xanthus*, a third component can be found associated with the secretin subcomplex. This protein is called secretin-associated protein (TsaP) and is believed to anchor the subcomplex to the peptidoglycan (134).

1.3.1.5 Alignment subcomplex proteins

In T4P systems of Gram-negative bacteria, additional proteins are required to stabilize and connect the secretin with the ATPase-platform protein complex (135, 136). *Neisseria* and *P. aeruginosa* T4P systems encode four additional membrane-associated proteins (PilM, PilN, PilO and PilP) that collectively form an alignment subcomplex, which supports pilus polymerization (121). In *T. thermophilus* and *N. meningitidis*, it was suggested that PilM and PilN interact with each other, forming a ring on the cytoplasmic side of the inner membrane (137, 138). PilN further interacts with PilO creating a ring on the periplasmic side of the inner membrane. The PilN-PilO complex serves as link between the secretin and the ATPase-platform protein complex, thereby directly contributing to both pilus assembly and retraction (137, 139). In addition, the PilN-PilO dimer binds to the periplasmic inner membrane lipoprotein PilP, which interacts with the secretin pore (140, 141). Thus, the PillM-PilN-PilO complex functions both as an anchor of the whole alignment subcomplex in the inner membrane and through its interaction with PilP aligns the secretin. A similar interaction network among the components of the alignment subcomplex has been revealed by cryo-ET of the T4P machinery in *M. xanthus,* suggesting that these interactions are conserved among Gram-negative T4P expressing bacteria (120).

Moreover, *in vitro* reconstitution experiments in *N. meningitidis* have recently shown that the alignment complex (PilM-PilN-PilO-PilP) together with the assembly ATPase, the platform protein, the major pilin and the prepilin peptidase, form

the minimal essential machinery required for pilus assembly in this pathogen. According to the authors, this subcomplex is thus a key piece of the assembly apparatus rather than just an alignment complex (135).

Finally, homologues of PilM, PilN and PilO have also been predicted in Gram-positive bacteria including *Clostridium perfringens*. However, their role and function in bacteria lacking an outer membrane is elusive. PilM-PilN-PilO proteins in *C. perfringens* were predicted to have similar membrane topologies as the protein homologues in Gram-negative bacteria (65). Thus, it was speculated that these proteins are likely to interact with yet unknown partners to form a complex that might allow pilus extension through the cell wall. The lack of an outer membrane in Gram-positive bacteria might also explain why PilP, which interacts with the secretin in Gram-negative bacteria, is absent in these systems (65).

1.3.2 Type IV pili assembly (and disassembly) mechanism

Having discussed the individual components required to assemble T4P, the next section will describe the details of T4P biogenesis using *Neisseria* spp. as example. Thus, the nomenclature of the pilus components present in *Neisseria* will be used herein.

The core T4P machinery in *Neisseria* can be divided into four major subcomplexes: (i) the outer membrane secretin subcomplex formed by the secretin PilQ, the pilotin PilW and secretin-associated protein TsaP; (ii) the alignment subcomplex (PilM, PilN, PilO and PilP); (iii) the motor subcomplex composed of the platform protein PilG and the assembly ATPase PilF and the retraction ATPases PilT, PilU and PilT2 and finally (iv) the pilus filament itself formed by the major pilin PilE and additional minor pilins. The minor pilins include PilH, PilI, PilJ, PilK and PilL, which are important for pilus assembly and function, and PilV, PilX and ComP, which are dispensable for pilus assembly but crucial for pilus functions (63). Prior to T4P formation prepilin subunits are produced in the cytoplasm and secreted via the SecYEG pathway into the inner membrane (142). Initially, the prepilins remain as bitopic proteins anchored to the inner membrane where they are cleaved by the prepilin peptidase PilD (109). Subsequently, mature pilins are recruited to the platform protein PilG that is located in the inner membrane and the initiation side for pilus assembly. The energy to initiate pilus assembly is provided by the ATPase PilF through the platform protein PilG, where pilus polymerization occurs (136). Proper fibre elongation is ensured by the PilM-PilN-PilO-PilP subcomplex which connects the motor subcomplex and the secretin PilQ through which the pilus can cross the outer membrane (141). In *N. gonorrhoeae,* a pilus tip-located adhesin (PilC) is involved in adhesion to host cells (143, 144). After pilus assembly is completed, pilus retraction occurs by the action of the main retraction ATPase

PilT, which drives the disassembly of the pilus core (104). The PilT paralogue PilT2 enhances the speed of pilus retraction by supporting the function of PilT (122, 127). Finally, PilU was suggested to modulate the dynamic regulation of T4P-mediated microcolony formation and dispersal (145). A schematic representation of pilus assembly and retraction mechanism is described in Figure 2.

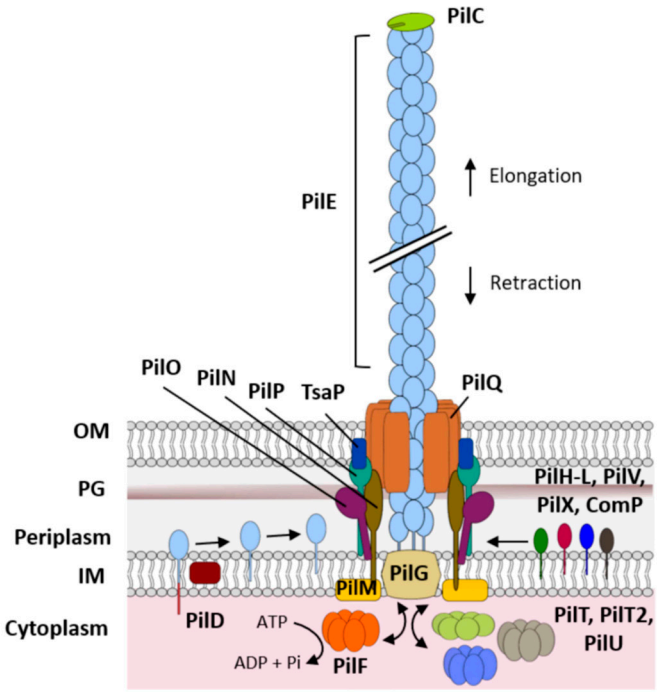

Figure 2. General organization of type IV pili in *Neisseria* spp. Adapted and modified from (63, 121). OM - outer membrane, PG - peptidoglycan and IM - inner membrane.

1.3.3 Type IV pili-associated functions

T4P are extremely versatile structures, which can mediate a wide range of functions. In this section, a general overview of the most common functions including adhesion, microcolony and biofilm formation, twitching motility, DNA uptake as well as other functions will be discussed.

1.3.3.1 Adhesion

T4P are common key virulence factors of many pathogenic bacteria as they promote their adhesion to host cells leading to colonization and establishment of severe infections. T4P facilitate adhesion in various ways as opposed to other types

of pili, which typically exhibit a single pilus component with intrinsic adhesive features at their tip. For gastrointestinal pathogens, such as EPEC and *C. difficile,* it was shown that adhesion can be mediated by the major pilin itself. The major pilin of EPEC is called bundlin and functions as adhesin (lectin) with affinity for N-acetyllactosamine receptors on human intestinal cells (146). The Gram-positive pathogen *C. difficile* also binds to human intestinal cells via T4P contributing to persistence in the host intestine. Interestingly, mutants lacking the major pilin PilA1 were able to establish initial colonization but they were outcompeted by the WT strain in long-term intestinal colonization in a mouse model (147). These examples highlight that T4P-mediated adhesion is a feature shared by both Gram-negative and Gram-positive bacteria. In fact, expression of the major pilin PilA2 of *C. perfringes* by a *N. gonorrhoeae* strain lacking its native major pilin PilE resulted in the assembly of functional pili, which adhered to mouse myoblast cell lines (148). This is an interesting representation of the level of conservation of T4P systems and its related functions among Gram-negative and Gram-positive bacteria.

Non-pilin proteins with adhesive properties present at the pilus tip of human pathogens like *Neisseria* spp. or *P. aeruginosa* have also been implicated in T4P-mediated adhesion (143, 149, 150). The adhesin PilC in *Neisseria* spp. plays a central role in adhesion to human epithelial cells (144). The functional display of PilC in the filaments requires the core minor pilins PIlH-L and the accessory minor pilin PilV (105, 124, 151). PilV itself was shown to bind to the immunoglobulin superfamily member CD147 mediating adhesion of *N. meningitidis* to human brain or peripheral endothelial cells (152, 153). This leads to the formation of membrane protrusions around adhering bacteria thereby increasing their resistance to shear forces created by the blood flow (154).

1.3.3.2 Microcolony and biofilm formation

Microcolony and biofilm formation are survival strategies developed by bacteria to adapt and persist in strict environments. The formation of microcolonies requires bacterial self-association and represents one of the initial steps in host colonization that later often leads to formation of mature biofilms associated with chronic infections (155). Microcolony formation is therefore closely associated with the ability of many pathogens to adhere to host cells. Thus, like adhesion, microcolony formation can be mediated by T4P, namely by pilus subunits like the major pilin or minor pilins present in the fibres (156, 157). In *N. meningitidis*, the accessory minor pilin PilX shows intrinsic aggregative properties and promotes bacterial auto-aggregation by counteracting the retraction activity of PilT to originate stable aggregates (157). In addition, *Neisseria* T4P systems present two PilT paralogues (PilT2 and PilU), which support the function of the main retraction ATPase PilT. The loss of PilU in *N. meningitidis* resulted in a delayed microcolony formation during infection. Since no major morphological differences were

observed between T4P of the *pilU* mutant and the WT, PilU seems to be able to mediate the proper display of pilus components involved in adhesion (145). In *N. gonorrhoeae*, correct gonococcal microcolony formation after adhesion to host cells is influenced by the retraction levels of PilT in which *ΔpilT* mutants formed less microcolonies and were less infective (158). This represents an interesting synergy between two T4P-related functions, adhesion and microcolony formation, in enhancing both meningococcal and gonococcal pathogenicity.

The ability of T4P to aggregate laterally forming bundles also triggers bacterial aggregation and microcolony formation (159, 160). For example, *V. cholerae* TCP self-aggregate through lateral pilus interactions mediated by the major pilin, bringing bacteria together to form microcolonies, which protect them from host defences (159, 161).

Bacterial microcolonies can develop into mature biofilms to enhance bacterial protection against host immune defences and external factors, such as antibiotic treatment. Biofilms are crucial in chronic infections caused by *P. aeruginosa* and T4P have been shown to play a key role in both microcolony and biofilm formation of this pathogen (162). In the absence of the retraction ATPase PilT, non-retractile mutants exhibited irregular biofilm structures indicating that the architecture of *P. aeruginosa* mature biofilms is influenced by T4P-driven bacterial motility (163, 164). T4P were also shown to be required for microcolony and biofilm formation in Gram-positive pathogens such as *Clostridia* spp. as T4P-deficient mutants formed substantially less biofilms than the WT strain (165, 166).

1.3.3.3 Twitching motility

Twitching motility is a flagellum-independent form of motility adopted by bacteria to move over moist surfaces and is commonly observed in bacterial aggregates with cell-to-cell interactions (167). Twitching motility allows bacterial communities to follow stimuli, such as gradients of chemical attractants or nutrient availability, enabling them to colonize new niches. This type of locomotion is driven by the extension, binding, and retraction of T4P located at bacterial poles (121). Bacteria extend T4P localized towards the stimulus allowing them to adhere to the surface. Then, pili retraction powers twitching motility with the motor force generated by pulling pili exceeding the force (≈50pN) required to detach other T4P from the surface (168).

Twitching motility helps human pathogens to move from one site of infection to another. For instance, *P. aeruginosa* mutants lacking the retraction ATPase are nonmotile and exhibited reduced virulence associated to the inability to translocate across the corneal epithelium and to disseminate (169). Interestingly, a study using microfluidics devices to mimic the biophysical properties of the human vasculature, such as shear stresses and flow patterns, revealed that

P. aeruginosa uses T4P to move upstream. This likely represents a competitive advantage for this pathogen to escape faster-growing bacteria and colonize new sites (170). In addition, it was recently reported that intracellular *P. aeruginosa* relies on twitching motility to disseminate throughout the cytoplasm of host cells after vacuolar escape, strengthening the role of T4P-mediated motility in *P. aeruginosa* virulence (171).

This type of motility also plays an important role in *N. gonorrhoeae* pathogenesis as motility of gonococcal communities is dependent on coordinated T4P retraction to successfully migrate on human epithelial cells (172). Interestingly, the activity of PilT2 enhances the twitching speed of gonococcal microcolonies supporting the idea that PilT parologues play a role in fine-tuning T4P dynamics (122).

Gram-positive pathogens, such as *S. sanguinis* and *C. difficile,* also use twitching motility as form of locomotion, and mutations in the ATPase resulted in non-motile aggregates or in aggregates with diminished motility, respectively (173, 174). In *S. sanguinis,* it was further observed that deletion of an uncharacterized gene named *pilK* (with no homology to *pilK* in *Neisseria*) resulted in abnormal bacterial motility patterns through an yet unknown mechanism (173).

1.3.3.4 DNA uptake

A subset of bacterial species is competent for natural transformation. Among those, some are known to take up DNA from the environment by a preserved mechanism involving the assembly of T4P (27). These filaments are assembled in the early steps of natural transformation and are essential for DNA uptake (25). The exact mechanism through which T4P bind and drive DNA uptake remains one of the major questions in the field. This process may vary between species and for this reason different models of T4P-mediated DNA uptake have been proposed which includes DNA binding by major pilins or alternatively the presence of minor pilins in the filament which can act as DNA receptors (175). Conceptually, DNA binding by the major pilin would represent the simplest way considering that it is the primary pilus component. However, direct evidence that major pilin monomers have DNA binding properties are still lacking. It is important to note that while the monomer or recombinant protein does not bind DNA, the properties of major pilins assembled into pili may differ from the monomers, creating a surface that could promote DNA-binding.

More robust evidence supports the presence of DNA receptors in the filaments as explanation for DNA binding. In *Neisseria* spp.*,* the accessory minor pilin ComP has intrinsic DNA-binding ability, recognizing DNA uptake sequences (DUSs), which allow bacteria to take up primarily species-specific DNA (176, 177). Among other transformable bacteria, *H. influenzae* also shows a bias to take up DNA by recognizing species-specific sequences called uptake signal sequences (178).

Recently, live fluorescence microscopy imaging revealed that T4P assembled by competent *V. cholerae* are able to bind DNA presumably through minor pilins (VC0858 and VC0859) present at the tip of the pilus (179). DNA binding at the pilus tip seems to be compatible with the notion that many T4P retract, bringing the DNA to the cell surface where it can be internalized. In fact, blocking pilus retraction in *N. gonorrhoeae* and *V. cholerae* has a drastic impact on DNA uptake resulting in non-transformable bacteria or in very low transformation rates (125, 179). In both pathogens, T4P retraction is concomitant with the accumulation of a periplasmic DNA-binding protein (ComE in *Neisseria* and ComEA in *Vibrio*) at the site of DNA uptake (179, 180). Compelling observations, using optical tweezers in *N. gonorrhoeae*, support DNA uptake by a translocation ratchet model. In this model, pilus retraction brings the DNA to the secretin pore in the outer membrane from where the DNA diffuses towards the periplasm where it binds to ComE acting as a chaperone (181). How DNA in Gram-positive bacteria is translocated across the thick peptidoglycan layer remains an intriguing question. In Gram-negative bacteria, the secretin ensures pilus retraction and subsequent DNA translocation across the outer membrane, but secretin-homologues forming a similar pore in the cell wall of Gram-positive bacteria have not yet been identified.

1.3.3.5 Other functions

The extraordinary versatility of T4P goes beyond the most widespread features described above. For instance, environmental bacteria belonging to the genus *Geobacter* assemble T4P with conductive properties (182). In these bacteria, T4P function as nanowires to transfer electrons involved in the reduction of iron oxide minerals and uranium (182, 183). This unusual T4P feature has inspired new approaches to engineer novel conductive biomaterials with possible application in clinical settings. The development of peptide-based coatings using peptides derived from PilA, the major pilin of *P.* aeruginosa T4P, has shown promising effects in preventing corrosion of metallic materials including stainless steels which are commonly used in medical devices and implants (184). In addition, adhesion of bacteria to stainless steel surfaces coated with these peptides was reduced which can be an interesting approach to tackle biofilm formation (185).

Other less common T4P associated functions can be found in cyanobacteria, which use T4P to sense and move towards the light in a process called phototaxis. This allows them to produce their own “food” via photosynthesis and represents a crucial survival mechanism for these bacteria (186).

1.4 Type IV pilins: structural insights into the building blocks of T4P

In general, pilins are small proteins ranging from 7 to 20 kDa in size. Most type IV pilins share a similar domain structure but can differ substantially in size and sequence (107). Structurally, the majority of pilin proteins characterized to date resemble a "lollipop", with a globular C-terminal domain and an extended N-terminal domain (α1) (Figure 3). Half of the extended N-terminal domain extends into the C-terminal domain (α1-C) and the other half (α1-N) protrudes from the globular C-terminal domain forming the "lollipop stick" (107, 108). The N-terminal domain is highly conserved and formed by a α-helix, whereas the C-terminal domain is typically composed of both α-helices and β-sheets (108). The conserved α1-N domain acts both as transmembrane domain and as protein-protein interaction domain, anchoring the globular domain in the inner membrane prior to pilus assembly. In contrast, the αβ-loop region in the C-terminal end contributes to the structural and functional diversity associated with these proteins (187). The α1-N region is highly conserved and hydrophobic with the exception of the glutamate residue at position 5 (E5) after the prepilin peptidase cleavage site whereas the α1-C region is hydrophilic. E5 in mature pilins has been shown to be dispensable for processing but important for pilus assembly, as mutants in E5 cannot assemble T4P (188, 189). This particular residue was proposed to mediate the docking of pilins during pilus polymerization by establishing electrostatic interactions with the N-terminus of the last pilin incorporated in the growing pilus (190). Finally, many pilins present two disulphide-bonded cysteine residues (D-region), which are important for protein stability. In order to assemble T4P, pilins are arranged in a helical array with their N-terminal domains forming the pilus core and their globular C-terminal domain creating the outer shell of the filament (191). The similar architecture between different type IV pilins reinforces the idea that the classical fold is conserved among these proteins (107, 108).

Most T4P are homopolymers formed by one major pilin. Notably, the recent structural analysis of *S. sanguinis* T4P revealed that these filaments are composed of a 4:3 ratio of two sequence identical major pilins, PilE1 and PilE2. Despite this distinct structure, the authors observed that major pilin processing and pilus assembly followed conserved mechanisms present in other bacteria (192).

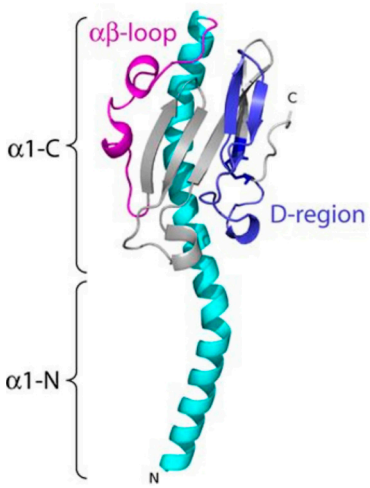

Figure 3. Structure of the *N. gonorrhoeae* major pilin PilE as representative example of a "lollipop-like" structure. Adapted with permission from Giltner et al., 2012. (107).

1.5 The human pathogen *Streptococcus pneumoniae*

The first observations of *S. pneumoniae*, also known as the pneumococcus, date to 1881 and are attributed to George Miller Sternberg and Louis Pasteur who independently isolated *S. pneumoniae* from rabbits injected with human saliva and from saliva of a rabies-infected child, respectively (193). Pneumococci are facultative anaerobic Gram-positive lancet-shaped bacteria, which typically grow in pairs as diplococci, but can also occur in single cocci or in short chains. On blood agar plates, they form small (1-2 mm) and greyish α-haemolytic colonies (194). In addition, pneumococcal colonies can have a mucoid appearance due to the presence of a polysaccharide capsule, which coats the pneumococcal surface. To date, at least 98 different pneumococcal serotypes have been identified according to their capsule structure (195, 196). Three additional phenotypic features allow identification of *S. pneumoniae.* Pneumococci are catalase negative, susceptible to optochin and bile soluble (197). Susceptibility to optochin has been broadly used as primary test to differentiate pneumococci from other α-haemolytic streptococci (198). However, reports of pneumococcal isolates resistant to optochin and of closely related species, including *S. mitis* and *S. pseudopneumoniae*, which are susceptible to optochin make this test alone not enough to accurately identify *S. pneumoniae* (199-201). Thus, alternative tests including bile solubility, presence of capsule or PCR techniques targeting genetic loci unique to pneumococci should be considered in some cases for the correct identification of *S. pneumoniae*, especially in the clinical setting (199, 202, 203).

Pneumococci are common colonizers of the human upper respiratory tract, however, when given the opportunity, they can cause infections, which range from mild mucosal infections, including otitis media and sinusitis, to life-threatening infections such as pneumonia, sepsis and meningitis (204). Historically, the pneumococcus has been a devastating pathogen tightly related to the massive number of deaths during the Spanish flu pandemic in 1918-1919. It is estimated that more than 50 million people died during the pandemic primarily due to co-infections with *S. pneumoniae* (205). Nowadays, pneumococci still represent a risk mainly for children under five years old and the elderly but also for immunocompromised persons. In 2015, it was estimated that pneumococcal infections were responsible for over 317 000 deaths globally among children under the age of five (Figure 4) (206).

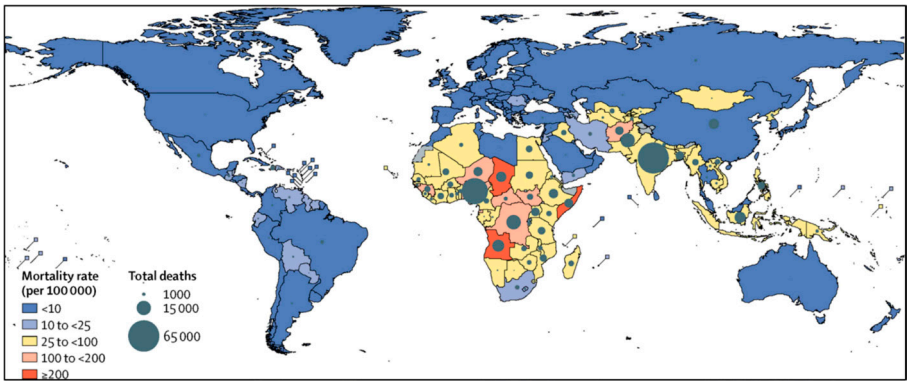

Figure 4. Global pneumococcal mortality rates and total number of deaths in HIV-negative children under the age of five in 2015 (206).

1.5.1 Pneumococcal colonization and disease

Pneumococcal infections occur through the spread of pneumococci-containing droplets or aerosols. However, successful transmission often results in asymptomatic colonization of the nasopharynx and carriage prior to infection. The upper respiratory tract therefore serves as a reservoir of pneumococci which is especially prevalent in pre-school age children attending day-care centres, with carriage rates up to 60% (204, 207). In the adult population, carriage rates are much lower (<10%) although higher colonization rates can be found in parents of small children (208, 209).

Pneumococcal disease involves the spread of pneumococci from the nasopharynx to other sites such as the middle ear and sinuses causing otitis media and sinusitis, respectively. These pathogens can also gain access to normally sterile sites of the body, including the lower respiratory tract, bloodstream or meninges, leading to invasive pneumococcal diseases (IPD) (204).

Acute otitis media (AOM) is an inflammatory disorder affecting the middle ear in which it is estimated that by the age of three, up to 80% of the children have been affected at least once (210). Although viruses are well-known causing agents of otitis media, they predispose individuals to bacterial AOM caused by pneumococci and other pathogens, including *H. influenzae*, and *Moraxella catarrhalis*, with *S. pneumoniae* accounting for 30-50% of AOM cases globally (211-213). Recurrent or persistent AOM can lead to more complicated conditions including hearing loss or perforation of the tympanic membrane. In Sweden and the USA, AOM was reported to be responsible for most of antibiotics prescription among children (214).

Sinusitis is an infection of the sinuses that is caused by viruses and bacteria. Bacterial sinusitis is common, and *H. influenzae* and *S. pneumoniae* are the most common pathogens isolated in paediatric sinusitis. Among those, patients infected with pneumococci present a more severe disease than those infected with *H. influenzae* (215).

The development of IPD during colonization is related to both host susceptibility and invasiveness of the pneumococcus (216). *S. pneumoniae* is the most common cause of community-acquired pneumonia (CAP). The 30-day mortality rate due to pneumococcal CAP vastly depends on the degree of severity of clinical signs but it can be as high as 20% in hospitalized patients (217-219). The distribution of pneumococcal CAP cases differs vastly between geographical regions and economical standards. In Europe, mortality rates vary from 1-48% depending on the country. However, a common denominator is that the mortality is much higher in patients ≥65 years of age (220, 221). Predominantly, pneumococcal CAP does not result in bacteremia, however, it has been reported that approximately a quarter of pneumococcal CAP cases can lead to septicemia with higher prevalence reported among older patients with comorbidities and male patients (222-225).

Finally, the most severe pneumococcal manifestation is meningitis, an acute inflammation of the meninges. Pneumococcal meningitis has high mortality rates, with 18-30% of patients dying. Among the survivors, around half suffers severe neurological sequelae, including hearing loss and cognitive deficits (226, 227). Among the risk groups, children are at greater risk where the global incidence rate is estimated to 17 per 100 000 children under the age of five. As for CAP, the mortality rate is strongly related with the economic situation of the country (228, 229).

1.5.2 *S. pneumoniae* morphology in relation to carriage and invasive disease

Pneumococcal nasopharyngeal colonization is regarded as a prerequisite for the development of disease. Colonization of the nasopharynx is a dynamic process where interspecies competition can affect or prevent the establishment of pneumococci in the nasopharyngeal flora (230). In addition, colonization requires that pneumococci successfully bind to the epithelial cells. Experimental evidence shows that the different morphologies adapted by *S. pneumoniae* can contribute to the ability to colonize or lead to invasive disease (231, 232). The ability of pneumococci to adhere to epithelial cells *in vitro* and to colonize in a murine model was shown to be related to long-chain forms. By using the clinically relevant encapsulated strain TIGR4 as model, it was shown that chain-forming mutants adhered and colonized better compared to the WT (232). The morphological heterogeneity presented by *S. pneumoniae* may contribute to its ability to establish as commensal or pathogenic bacteria. Longer chains can facilitate adherence by providing a larger contact area with the host cells, whereas the subpopulation of single cocci or diplococci could be better to invade due to their smaller size. Consistent with this, it was shown that minimizing bacteria length conferred a competitive advantage in a systemic infection mouse model (231).

S. pneumoniae can be killed via opsonophagocytosis by neutrophils. This involves opsonization of pneumococci by the complement system and deposition of the complement component 3 (C3) which can interact with the complement receptor on neutrophils (233). While shorter chain-forming pneumococci were shown to escape killing via opsonophagocytosis, longer chains promoted C3 deposition on the bacterial surface and were therefore more susceptible to phagocytosis by neutrophils (231). *In vivo,* mutants with increased chain length were attenuated due to complement-dependent clearance. In addition, another study observed the presence of only small bacteria size (single cocci) in the brain in a bacteraemia-derived meningitis mouse model. It was suggested then that the small size of single cocci could be beneficial to cross the blood-brain barrier into the brain and to cause meningitis (234). Thus, despite the fact that *S. pneumoniae* characteristically grows in diplococci, the presence of single cocci and chains may also influence the ability of various strains to colonize or cause invasive disease (Figure 5).

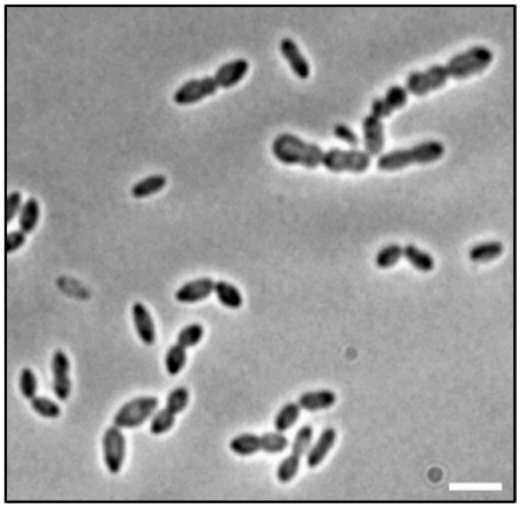

Figure 5. Phase-contrast micrograph of *S. pneumoniae* R6 grown on a blood agar plate overnight showing different morphologies. Scale bar 2µm

1.5.3 The pneumococcal capsule

The vast majority of pneumococcal clinical isolates are encapsulated, however, non-encapsulated strains can also be found, either because they have a mutation in the capsule (*cps*) genes or entirely lack the *cps* locus (235). The virulence of *S. pneumoniae* is mainly attributed to its polysaccharide capsule which surrounds the bacterium and protects it from the host immune system. Consequently, the capsule has been the target in the development of current pneumococcal vaccines (discussed in the next section). The thickness of the capsule varies among the 98 different serotypes identified to date and it can be up to 400 nm thick in some serotypes (236). In addition, the composition also differs substantially among them, which influences the pneumococcal potential to colonize or cause invasive disease (237-239). In Stockholm county, a study which characterized the effects of pneumococcal conjugated vaccines in carriage and invasive disease, observed that the vaccine serotypes 3 and 19A and the non-vaccine serotypes 22F and 23A showed the greatest increase among IPD cases after vaccine introduction in the childhood vaccination program, however, serotypes 11A and 35F were more prevalent in childhood carriage (240). Most pneumococcal capsular serotypes are negatively charged which contributes to the overall surface charge (241). The electrostatic repulsion with host immune cells facilitates pneumococci to escape from the mucus-mediated clearance and phagocytosis (241, 242). Non-encapsulated strains are typically less virulent in mouse models and only in rare situations they cause IPD in humans (243-245). However, non-encapsulated

strains *in vitro* are more prone to acquire genes through natural transformation than isogenic encapsulated strains (246). Taking *in vitro* observations into consideration, non-encapsulated strains have higher transformation frequencies. This may indicate that transformation can be facilitated among non-encapsulated strains in the nasopharynx in which the lack of capsule also enhances bacterial binding to epithelial cells.

1.5.4 Therapeutic options and prevention of pneumococcal diseases

Pneumococcal diseases are still a considerable cause of mortality and bring a heavy burden to healthcare systems. The treatment of choice used to tackle pneumococcal infections are antibiotics, namely β-lactam antibiotics (e.g. penicillin), macrolides (e.g. erythromycin) and fluoroquinolones (e.g. norfloxacin) (247). β-lactams target the bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs). However, mutations in pneumococcal PBPs or in the cell-wall muropeptide branching enzyme MurM have been involved in the development of pneumococcal resistance to penicillin and other β-lactams (248). The level of resistance varies largely between regions with certain areas in Asia reporting ≥50% of the isolates as being non-susceptible to penicillin (249). In Sweden, an official report from 2018 shows that the incidence of *S. pneumoniae* with reduced susceptibility to penicillin was approximately 1 case per 100 000 inhabitants, of which most cases (35%) were detected in young children of up to four years of age (250).

Macrolides, such as erythromycin, inhibit protein synthesis through binding to the 50S ribosomal subunit. In pneumococci, resistance to macrolides can arise by methylation of the macrolide target site by the *ermB* methylase or by active efflux of the drug through an efflux pump encoded by the *mefA* gene (251). Fluoroquinolones, like norfloxacin, are synthetically produced antibiotics, which inhibit topoisomerase enzymes (DNA gyrase) thereby promoting breakage of double stranded DNA. Pneumococci can develop resistance to fluoroquinolones by the action of the efflux pump encoded by *pmrA* or through mutations in the quinolone resistance-determining regions of the subunits of DNA topoisomerase IV *parC* and DNA gyrase *gyrA* genes (252, 253). Similar to penicillin resistance, pneumococcal resistance rates to macrolides and fluroquinolones also differ greatly depending on the country. In Sweden, 4.6% of invasive pneumococci isolated in 2018 were resistant to erythromycin and only 1.1% were resistant to norfloxacin (250).

To target serotypes predominantly associated with IPD cases, prevention plans were implemented with the development of pneumococcal vaccines (254). Vaccine

efficacy is mediated by the ability to trigger B-lymphocytes to produce protective antibodies and immunological memory. Currently, there are four different pneumococcal vaccines commercialized: the pneumococcal polysaccharide vaccine containing 23 capsular polysaccharides (PPSV23) and three pneumococcal conjugate vaccines with 7, 10 or 13 capsular polysaccharides conjugated to a carrier protein (PCV7, PCV10, PCV13). The list of serotypes covered by each vaccine can be found in Table 1. PPSV23 was the first vaccine to be introduced on the market in 1983. This vaccine stimulates an antibody production against the pneumococcal polysaccharide capsule through a T-lymphocytes independent immune response by direct activation of B-lymphocytes (255). The polysaccharide vaccine contributed to the reduction of IPD cases in adults, but they evoke no or only a weak immune response in infants younger than 2 years, one of the principal risk group for pneumococcal diseases (256). In young children, the spleen is not entirely developed hence B-lymphocytes are not fully matured. As a consequence, they do not respond to polysaccharides to evoke an immune response against pneumococci (257-259). There are also observations that the polysaccharide vaccine loses efficacy in the elderly as the function of B-cells weakens with age (260, 261). Thus, a polysaccharide-conjugated vaccine was developed to surpass the limitations of PPSV23, in particular to induce an immune response in young children. In 2000, PCV7 was the first conjugated vaccine to be introduced on the market, and PCV10 and PCV13 were licensed in 2010 (262). These vaccines are coupled to protein carriers (Table 1) and evoke an antibody response via a T-lymphocyte dependent response (263). In this response, B-cells closely interact with T-cells in the activation and production of antibodies via the carrier protein (264). PCV vaccines have revealed to elicit immune protection in young children as they can respond to T-cell dependent antigens. Upon introduction of PCVs in national vaccination programs, the number of pneumococcal infections was reduced drastically. In Sweden, PCVs are included in the childhood vaccination plan since 2007 and in 2016, 97.1% of children by the age of two year were vaccinated against pneumococcal infections (265). As result, a considerable decrease in the numbers of IPD cases in vaccinated children was observed (240, 266).

Vaccination has effectively reduced the nasopharyngeal carriage and IPD rates caused by pneumococcal serotypes included in the vaccines. However, serotype replacement by serotypes not included in the vaccines has been observed in different reports. This raises the concern that vaccination selects against the serotypes included in the vaccines, which are then replaced by non-vaccine serotypes not included in the PCVs (240, 267, 268). In addition, the rise in colonization and infections with non-encapsulated pneumococci should also be seen with caution. Non-encapsulated strains have been associated with higher transformation frequencies *in vitro* which in biological conditions may facilitate the spread of antibiotic resistance and virulence genes (245).

Table 1. Serotypes covered by the pneumococcal vaccines available on the market (269, 270).

Vaccine	Protein carrier	Serotypes covered
PPSV23 (Pneumovax, Merck)	None	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F
PCV7 (Prevnar®, Pfizer)	Non-toxic variant of the diphtheria toxin	4, 6B, 9V, 14, 18C, 19F, 23F
PCV10 (Synflorix™, GSK Biologicals)	Non-typeable *H. influenzae* protein D, diphtheria or tetanus toxoid	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F
PCV13 (Prevnar™, Pfizer)	Non-toxic variant of the diphtheria toxin	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F

1.5.5 Natural transformation in *S. pneumoniae*

Frederick Griffith first described natural transformation in 1928 in *S. pneumoniae*. In a series of elegant experiments, he demonstrated that a non-virulent pneumococcal (non-encapsulated) strain could become virulent when co-inoculated with a heat-killed virulent (encapsulated) strain and injected into mice, suggesting that a transfer event had occurred between bacteria (271). Later, in 1944, Avery and colleagues proved that DNA was the transforming principle (272).

Since its discovery, the transformation process in *S. pneumoniae* has been extensively studied and can be divided into four steps. First, pneumococci need to respond to internal and external cues, which allow them to become competent. Once the competent state is activated, competent pneumococci express all the genes involved in DNA uptake, integration, and recombination into the genome. A subset of these genes encodes proteins required for the formation of the T4P and a dedicated DNA uptake machinery named transformasome. Both the T4P and the transformasome are fundamental for natural transformation. Third, competent pneumococci lyse susceptible non-competent neighbouring bacteria by secretion of hydrolases thereby providing the genetic material for transformation, in a mechanism that is likely simultaneous to pilus formation. Finally, transcription of competence genes shuts down leading to the termination of the competence period.

In the following sections, an overview of competence induction and regulation, leading to expression of the components involved in DNA uptake, will be described. A description of the fratricide mechanism used by competent pneumococci to target non-competent siblings and its implications will also be presented.

1.5.5.1 Competence induction and regulation

Pneumococcal competence is induced by a peptide pheromone, named competence-stimulating peptide (CSP), secreted by the bacteria. CSP induction represents a quorum-sensing signalling that allows bacteria to communicate at an intercellular level to monitor and control their own population density. In laboratory conditions, when CSP is externally added to a culture in early exponential phase, peak competence is reached 15-20 minutes after induction. Subsequently, competence gradually decays to nearly zero in about 40 min (273). During competence, *S. pneumoniae* expresses two distinct sets of genes, the early competence genes and the late competence genes, which are respectively regulated by ComE and ComX. ComE controls the expression of the ABC-transporter ComAB and the three-component regulatory system ComCDE crucial in the beginning of competence induction (Figure 6). CSP, encoded by *comC*, is synthesized as a precursor peptide (ComC), which is processed and secreted by the ComAB transporter (273, 274). Different CSP types, or pherotypes, have been identified in *S. pneumoniae* and also in other streptococcal species (275). Mature CSP is then sensed by the membrane-embedded histidine kinase ComD, which phosphorylates the cognate response regulator ComE (276). Phosphorylated ComE (ComE~P) induces the expression of the pneumococcal early competence genes, which include *comAB*, *comCDE, comX* and *comM.* By promoting the expression of the *comAB* and *comCDE* operons, ComE~P creates a positive feedback loop that ensures enough CSP is produced and thereby allowing a coordinated competence development throughout the population (277). ComX is the alternative sigma factor which controls the expression of several late competence genes involved in DNA uptake and homologous recombination (278). *comM* endodes the fratricide immunity protein which protects competent pneumococci from lysis (see below) (279).

Competence induction can also be triggered by different types of antibiotics, such as streptomycin and kanamycin (280). The competence-stimulating effect of streptomycin and kanamycin is related to their ability to increase the rate of translational decoding errors. In the situation when translation accuracy is high the serine protease HtrA supresses competence by degrading CSP. However, when translation accuracy is low, HtrA is overloaded with misfolded proteins and is not able to degrade CSP, which accumulates and leads to competence induction (281, 282). In addition, DNA-damaging antibiotics and compounds like mitomycin C were shown to induce competence by stalling DNA replication elongation, leading to an increase in the copy number of genes located close to the origin of replication, which include the *comAB* and *comCDE* operons. As result, the levels of CSP increase and competence is induced (283). Recently, another study found that the β-lactam aztreonam and the β-lactamase inhibitor clavulanic acid induce competence by targeting the D,D-carboxypeptidase penicillin-binding protein 3 (PBP3) which phenotypically led to pneumococcal chain formation. This resulted

in retaining of CSP on the bacterial surface rather than its diffusion, facilitating the local CSP recognition by its membrane-bound receptor ComD. As a consequence, competence is not induced simultaneously in the entire bacterial population but rather gradually, which results in an overall longer competence period (284).

The shutdown of competence can also occur at multiple levels. The late competence protein, DprA plays an important role in shutting down competence by inhibiting ComE-driven transcription whereas the protease HtrA selectively degrades ComEA and ComEC, suggesting its participation in the termination of pneumococcal transformation by breakdown of the DNA uptake apparatus (285-287). A second layer of control to shutdown competence is proposed to occur through the competition between the alternative sigma factor ComX (σ^X) and the main sigma factor RpoD (σ^A) for the interaction with ComW and/or RNA polymerase binding by a yet unknown mechanism (288, 289).

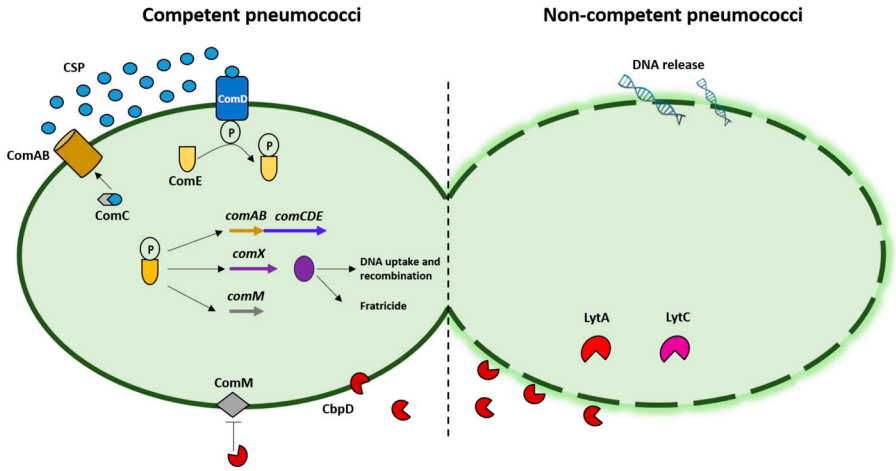

Figure 6. Schematic representation of competence induction in *S. pneumoniae*. Competent pneumococci synthesize the precursor peptide ComC which is processed into CSP. The ComAB transporter secretes mature CSP to the exterior, which is sensed by the histidine kinase ComD. Then, ComD phosphorylates the response regulator ComE, which induces the expression of *comAB, comCDE, comX* and *comM.* ComX regulates the expression of the late competence genes involved in DNA uptake and recombination and the fratricide genes. The fratricide immunity protein ComM protects competent pneumococci from the proteolytic activity of CbpD. Non-competent pneumococci are lysed by the activity of CbpD which activates the activity of LytA and LytC to enhance the lysis during fratricide. Lysed pneumococci release DNA that can be used as substrate in natural transformation (290).

1.5.5.2 Uptake and processing of transforming DNA

Upon competence induction and expression of early competence genes, *S. pneumoniae* expresses several late competence genes encoding the components required to form the DNA uptake machinery, also known as the transformasome (278). In addition, competent pneumococci assembly a 2-3 µm long pilus (discussed in the next section) that facilitates initial double strand DNA (dsDNA) binding and then directs it to the membrane-associated DNA receptor, ComEA (291). Upon binding to ComEA, the non-transforming DNA strand is degraded by the membrane surface endonuclease EndA. Interestingly, EndA is not regulated by ComX and is also expressed in non-competent pneumococci where it was shown to be involved in virulence by degrading neutrophil extracellular traps (NETs) (292). These traps are composed of both DNA and antibacterial proteins and are released by activated neutrophils (293). Thus, the degradation of NETs by EndA allows the release of captured pneumococci and enables their dissemination (292). In non-competent bacteria, EndA is localized uniformly in the membrane, however, during competence, EndA is recruited to the midcell by ComEA. This strongly indicates that the uptake of DNA takes place at this position in pneumococci, which coincides with the site of peptidoglycan synthesis (294). The spatial coordination between peptidoglycan synthesis and the assembly of the transformasome may facilitate the uptake of DNA through the cell wall.

The resulting single strand DNA (ssDNA) is then transported into the cytoplasm in the 3´ to 5´ direction through the channel formed by the transmembrane protein ComEC, with the assistance of other proteins, ComFA and ComFC (294-296). ComFA binds to ssDNA controlling the uptake rate and has ssDNA-dependent ATPase activity. In addition, it forms a complex with ComFC, which interacts with other transformasome components. This places ComFA and ComFC as the link between DNA uptake and DNA recombination during transformation (296).

Upon entry into the cytoplasm, ssDNA fragments are coated by the single-stranded DNA-binding protein B, SsbB, which protects them from degradation. In addition to bind to ssDNA, SsbB facilitates RecA-mediated recombination, and creates a pool of SsbB-ssDNA complexes that can undergo multiple recombination events. This last role stimulates chromosomal transformation and contributes to the genetic plasticity of *S. pneumoniae* (297). Mutations in this gene have been observed to negatively impact transformability showing the importance of SsbB in transformation (297).

Then, the DNA processing A protein, DprA, replaces SsbB and promotes the loading of RecA onto the ssDNA (298). RecA plays a crucial role in the search for homology and in the strand-exchange reaction allowing the incorporation of the transforming DNA into the recipient genome (299). Assuming that the incoming

ssDNA presents sufficient homologous regions with the genome of the recipient bacteria, homologous recombination will occur. Additional competence-specific proteins, such RadA and CoiA, also favour transformation. RadA is a DNA helicase, which interacts with RecA and is loaded onto ssDNA. Subsequently, it unwinds the parental duplex DNA and translocates along the transforming ssDNA, facilitating its recombination (300). The function of CoiA is still not fully understood (301). General organization of the DNA uptake machinery is described in Figure 7.

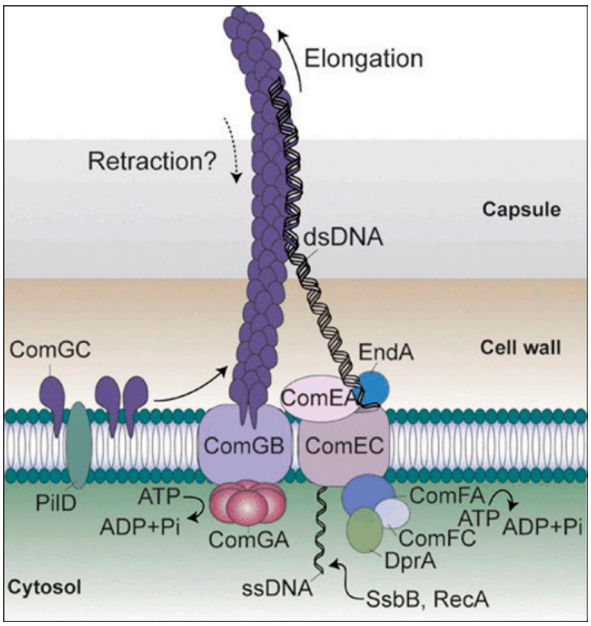

Figure 7. Schematic representation of the components involved in DNA uptake and homologous recombination in *S. pneumoniae*. Used with permission from Muschiol et al. 2019 (302).

1.5.5.3 The *comG* operon

During competence, *S. pneumoniae* also expresses the *comG* operon, a set of late competence genes regulated by ComX. This operon is composed of seven genes, namely *comGA*, *comGB*, *comGC*, *comGD*, *comGE*, *comGF*, and *comGG*. All of them are required to express type IV pneumococcal competence pili on the bacterial surface, which are essential for transformation (278). The first gene of the operon, *comGA*, encodes a putative secretion ATPase responsible for providing the energy for pilus assembly. Inactivation of *comGA* leads to non-piliated pneumococci (291). The next gene, *comGB*, encodes an integral membrane protein ComGB, thought to form the base structure. The remaining genes in the operon,

comGC-G, encode five pilin proteins. ComGC is the major pilin and it was shown to constitute the backbone of the competence pilus which can be 2-3 µm in length (291). Pneumococcal strains lacking *comGC* are non-piliated and non-transformable, suggesting that competence-induced pili are essential for transformation. DNA binding experiments suggested that the pneumococcal competence pilus functions as the primary DNA receptor on the surface of competent pneumococci (291). *comGD, comGE, comGF* and *comGG* encode four minor pilins whose role in pilus assembly and transformation remains elusive and investigating their role in DNA uptake is one of the aims of this thesis. The processing of the pneumococcal pilins is performed by the prepilin peptidase PilD, which is encoded by the *pilD* gene located elsewhere in the genome (303).

1.5.5.4 Fratricide - the killing mechanism

Competent pneumococci can target and acquire genetic material from non-competent siblings. In bacterial cultures, this killing mechanism, named fratricide, allows the subpopulation of competent pneumococci to use the genetic material released as substrate in natural transformation (279).

The product of the early competence gene *comM* and of the late competence genes *cbpD*, *lytA* and the σ^A-dependent gene *lytC* have been described as direct players in fratricide. Lysis is accomplished by the activity of the autolysin LytA, the lysozyme LytC and by the key fratricide cell wall hydrolase CbpD (24, 304). CbpD was shown to be crucial for fratricide and is also active against closely related species including *S. mitis* and *S. oralis* (304-306). In addition, it promotes the activity of lytic enzymes LytA and LytC in the target cells thereby stimulating and increasing the efficiency of lysis of non-competent bacteria. However, in contrast to CbpD, LytA and LytC, are not essential for fratricide (304, 306). Competent pneumococci protect themselves against self-lysis by expressing an immunity protein named ComM, which is encoded by an early competence gene [55, 61]. As result, the DNA released by non-competent siblings becomes available to be used in natural transformation by competent pneumococci. Thus, competence-induced fratricide may facilitate DNA repair through the transfer of homologous genetic material from non-competent pneumococci. Interestingly, *in vitro* studies suggest that DNA released during fratricide within biofilms leads to enhanced exchange of genetic material between pneumococci (307). In nature, it may favour gene transfer between closely related species in multispecies biofilms in the human nasopharynx allowing *S. pneumoniae* to adapt and survive in this environment.

1.5.5.5 The role of competence in pneumococcal pathogenesis

ComE and ComX control the two waves of gene expression observed during competence. ComE up-regulates a subset of early competence genes and ComX controls the expression of at least 80 late competence genes (278, 308). Interestingly, only 23 genes were required for transformation of *S. pneumoniae* in laboratory conditions (278). A systematic deletion analysis of ComX-regulated genes identified 14 genes that were implicated in bacteremia and/or acute pneumonia in competitive or single mouse infection models (309). Among those, genes involved in stress response, cellular metabolism, and transport were identified. Mutants lacking genes required for fratricide, such as *lytA, cbpD,* and the bacteriocins *cibA* and *cibB,* were also less virulent in single-bacteremia infections and less competitive than WT in causing bacteremia and acute pneumonia *in vivo*. In addition, deletion of essential genes for genetic transformation including *dprA* and *recA* drastically reduced virulence in bacteremia and acute pneumonia models (309). Finally, *in vivo* competition assays of each late competence gene mutant in a ComX-null genetic background against the parental strain lacking ComX identified genes with basal expression levels (e.g. *lytA*) and competence-induced genes contributing to the bacterial fitness during infection (e.g. *dprA*) (309). In a recent follow-up study, it was shown that the Δ*dprA* mutant had a dysregulated overexpression of proteins involved in fratricide including LytA, CbpD and ComM. Overexpression of LytA and CbpD caused a higher susceptibility to lysis in the Δ*dprA* mutant *in vitro*. High levels of ComM led to growth arrest and reduced virulence in a mouse model of acute pneumonia. In addition, the inability of the mutant to shutdown competence was suggested to represent an energetic burden to the bacterium. Altogether, it was proposed that the deletion of DprA caused competitive disadvantages, which resulted in pneumococcal virulence attenuation *in vivo* (310). Loss of function in the early competence genes *comB* and *comD* also decreased the ability of *S. pneumoniae* to cause invasive diseases in mice (311, 312). Moreover, ComE was shown to negatively regulate the transcription of genes involved in the production of capsule in *S. pneumoniae* D39. Thus, deletion of *comE* led to increased transcription of capsule genes leading to a decrease in colonization fitness and enhanced virulence in adult mice models (313). In contrast, another study reported that deletion of ComE facilitated colonization in a newborn rat competitive colonization model of asymptomatic carriage (314).

Analysis of the gene expression profiling during infection (pneumonia and sepsis) *in vivo*, showed that the competence genes *comA, comE* and *comX* were found to be less expressed during sepsis than in pneumonia conditions (312). Interestingly, it was also observed that the gene expression profiling of bacteria growing in biofilms *in vitro* was similar to the bacteria recovered from mice with pneumonia. In contrast, bacteria grown in liquid cultured presented the same pattern of expression as bacteria recovered from septic mice. It was therefore suggested that

competence is involved in the regulation of distinct bacteria growth phenotypes, depending on the site of infection (312).

Biofilm formation plays an important role in pneumococcal pathogenesis being involved in the transition from asymptomatic colonization to spread and disease (315). Recently, a new ComE-induced gene, named *briC*, was shown to encode a peptide which promotes biofilm formation *in vitro* and nasopharyngeal colonization *in vivo* (316). Genetic exchange and the transformation efficiency were shown to be enhanced in biofilms growing *in vitro* under mimicking the conditions in the nasopharynx such as temperature and interaction with epithelial cells *in vitro.* In addition, co-colonization of mice with two pneumococcal strains resulted in higher transformation frequencies compared to a sepsis model (246).

Considering the importance of key competence proteins in pneumococcal virulence, targeting these elements has been considered as a potential approach to control pneumococcal infections (317). Modified CSP peptides were reported to competitively inhibit competence and gene transfer as well as to attenuate virulence of *S. pneumoniae in vivo* (318). Recently, a high-throughput screen identified inhibitors of pneumococcal competence, that block the proton motive force. When these inhibitors were used, the CSP export was disrupted which prevented competence and natural transformation *in vitro* in an epithelial colonization model and also reduced HGT in a mouse infection model (319).

1.6 Microfluidics and label-free particle sorting techniques

Microfluidics is defined as a distinct area of research involving fluid manipulation in channels at the micrometre scale. This research area is relatively new with its origins towards the end of the 20th century. However, it has quickly emerged as a promising field considering its application in many other research areas such as biological, medical, and physical sciences (320). The vast range of available microfluidic techniques allows its use from fundamental research to diagnostics. A key concept related to microfluidics is the possibility to develop lab-on-chip devices, which integrate different microfluidics principles with biological or chemical assays. Due to their portability and small dimensions, these devices provide the possibility of using small volumes and handling a large number of samples in a well-controlled manner and with high level of sensitivity. This allows a reduction of the overall costs and it shortens the time of experiments. However, a potential limiting factor is that the creation of such devices also requires the development of advanced fabrication techniques (321).

The implementation of microfluidic techniques in the microbiology field has allowed us to study bacterial dynamics with further depth. Droplet microfluidics techniques have allowed investigations of phenotypic and genotypic variations in

E. coli subpopulations for long periods of time. Individual bacteria were encapsulated in droplets after a period of starvation and the development of each derived subpopulation was followed and characterized over time (322). Interestingly, another study using droplet microfluidics created a method to perform high-throughput screening of antibiotic-resistant bacteria. This method allowed sorting and identification of mutants resistant to antibiotics by creating droplets where bacteria were grown in the presence of the bacteriostatic antibiotic fusidic acid. Only bacteria resistant to the antibiotic could proliferate and were then sorted based on their light-scattering properties (323). Moreover, a herringbone-type microfluidic platform containing an antimicrobial peptide on its surface was used to isolate *Salmonella* and *Brucella* spp. from large volumes (10 ml) of urine samples with a high-sensitivity degree (5 cfu/ml and 10 cfu/ml, respectively) (324). These examples highlight the importance and usefulness of designing microfluidic devices to study bacterial populations or to be applied in diagnostics.

One area which gained particular interest within microfluidics due to its numerous biological applications is particle sorting. Cell sorting using microfluidics devices can be grouped into two categories: labelled techniques and label-free techniques. The first group of techniques exploit the use of labels or antibodies, which can be coupled to the surface of the devices and be used to separate and enrich particles. This type of microfluidics techniques has been widely used in tumour biology and showed a high degree of sensitivity in the detection and sorting of circulating tumour cells (325-327). The second group of techniques exploits intrinsic physical properties of the biological particles to separate, without the use of any type of label. This represents a great advantage compared to conventional sorting techniques such as flow cytometry or fluorescence activated cell sorting (FACS), allowing the recovery and use of the sample for downstream experiments (328).

Microfluidic sorting, which uses label-free sorting and separation techniques can be further sub-grouped into two classes: active or passive methods. Active microfluidic techniques involve the application of external fields such as electric, magnetic or acoustic fields to sort particles according to their size and morphological properties. Examples of active techniques for particle sorting include acoustophoresis, electrophoresis and optical tweezers (329). In contrast, passive microfluidic methods do not require external force fields as they take advantage of intrinsic fluidic properties such as inertia or viscosity and also consider interactions between particles in suspension. Some of the techniques included in this category are inertial microfluidic separation (iMF), pinched flow fractionation, and deterministic lateral displacement (DLD) (329). As the name suggests, iMF uses the inertial forces of particles to be sorted combined with the flow rate of the buffer where the particles are resuspended in. This technique has been used to

sort bacterial suspensions with bacteria migrating differently according to their size along the microchannels of the device (330). In pinched flow fractionation, particles are sorted by being pushed against the wall of the device as a result of the forces created by the buffer flow rate. This technique considers that a (spherical) particle cannot be closer to the wall of the device than its radius, thus particles with different sizes can be separated (331). Finally, particle sorting using DLD is obtained by running samples through an array of posts that will separate the particles according to their size. A more detailed description of this technique will be presented in the following section.

1.7 Deterministic lateral displacement - general principles

DLD was first described in 2004, in a study in which the authors separated different spherical particles based on their size in continuous flow with a resolution down to 10 nm (332). Typically, DLD devices are one centimetre long, one millimetre wide and 10 µm deep (Figure 8A). Their composition is relatively simple and can be divided into three parts: (i) a set of inlet reservoirs, (ii) a pillar array, and (iii) a set of outlet reservoirs. The particle sorting involves the loading of the mixed sample to be sorted in one of the inlets and running buffer in the other(s). This will ensure the creation of a proper laminar flow along the device. Then, the mixed sample runs through the pillar array where they will be sorted according to their size. Finally, the sorted sub-populations can be collected from the different outlet reservoirs for further analysis (Figure 8B) (333).

The level of sorting efficiency strongly relies on the characteristics of the pillar arrays and the morphological properties of the particles. Each row of arrays is shifted from the previous row by a specific distance. This results in arrays that are not entirely aligned with the continuous flow which leads to the formation of different curvy streamlines that will allow particle separation (332). An important parameter that contributes to particle sorting is the critical diameter (DC) of the DLD device. The critical diameter of each device is calculated by using a specific formula, which takes into account the gap between two posts in a row and the row shift periodicity. Proper particle sorting is also affected by the depth of the device to avoid clogging, which is particularly important when sorting non-spherical biological particles like bacteria. These parameters combined with the size distribution of the particles will ensure the correct separation of the particles in the device. Particles smaller than the critical diameter remain aligned within their initial streamline without being displaced and they assume a “zig-zag” trajectory. In contrast, particles larger than the critical diameter will be displaced gradually along the device in a displacement trajectory ending up in a different position than the particles migrating in “zig-zag” mode (Figure 8C) (333, 334).

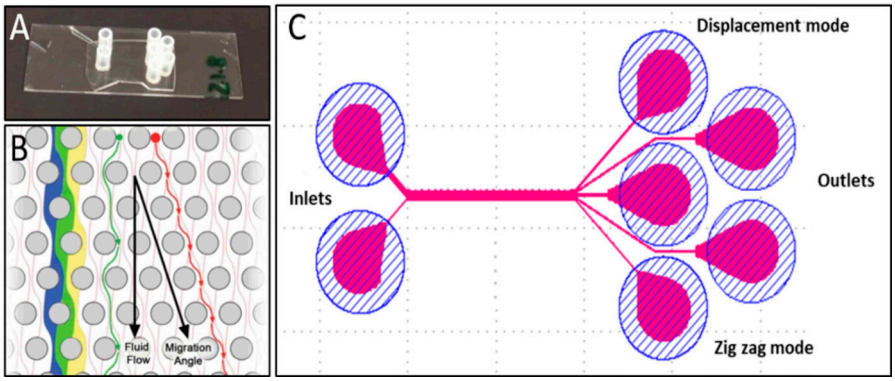

Figure 8. (A) Example of a DLD device used in this thesis. (B) Schematic representation of how a small particle (in green) and a larger particle (in red) migrate along the device. Adapted from (334) (C) Schematic representation of a DLD device with two inlets and five outlets used in this work, highlighting the outlets corresponding to the zig-zag mode and the displacement mode. Adapted from paper III.

1.7.1 Biological applications of DLD

DLD offers a large variety of throughput ranging from nanolitre per min (nl/min) to microlitre per minute (µl/min) which makes this technique an attractive solution to sort different types of biological samples. DLD devices have been used to sort white blood cells from red blood cells as well as circulating tumour cells from blood (335, 336). In addition, separation of parasites responsible for the human African trypanosomiasis (sleeping sickness) from human blood has also been accomplished by using this technique (337). Another promising prospect for DLD comes with the possibility to design and integrate devices to upstream and/or downstream applications. For instance, integrated high throughput devices using a combination of DLD arrays with an affinity-based technique allowed high yields of enrichment and capture of cancer cells from blood being an attractive approach for clinical cancer diagnosis (338).

1.7.2 DLD limitations

Despite being a flexible and versatile technique, there are also some inherent limitations. DLD has been designed based on specific events, such as particle diffusion, fluidic resistance and laminar flow, which become more prevalent at the microscale. In fact, diffusion and fluidic resistance are the two main challenges associated with the performance of DLD devices (333). Diffusion can disturb the laminar flow inside the device and may lead to particle mixing. For smaller particles running through the DLD devices, diffusion is usually residual and does not affect their trajectory. However, for larger particles diffusion may increase and affect

the separation efficiency. This can likely be overcome by a fine-tuned increase of the flow velocity. In addition, if the gap between the pillars is too small this may lead to additional fluid resistance in the device, which can make particles bind to the channel leading to device clogging (329). Clogging caused by the binding of biological particles to the surface of the device itself is also a matter of concern. Coating the devices with compounds that prevent particle-surface interactions but maintaining the intact morphology of the sample can overcome this problem.

Different modifications to the traditional DLD design have also been developed to minimize the limitations and to optimize the performance of the devices. Conventionally, the micropillars in the devices have a circular shape but a remarkable variety of different micropillar shapes have been designed such as rectangular, triangular, and I-shaped microposts aimed to reduce clogging problems and to increase sorting efficiency (333). Recently, airfoil-shaped posts have also been optimized to increase the throughput and sorting of soft and deformable particles (339). After the validation of these alternative designs, it will be exciting to see their performance to sort different types of biological samples.

2 AIMS

Natural transformation represents an important landmark in pneumococcal physiology and contributes to the remarkable genetic plasticity of this respiratory pathogen. It allows *S. pneumoniae* to acquire antibiotic resistances and evade vaccine pressure, making the infections caused by this pathogen hard to treat. Thus, understanding the underlying mechanisms of pneumococcal natural transformation can help us to identify alternative approaches to tackle this pathogen. A crucial step in natural transformation is the uptake of extracellular DNA, which in *S. pneumoniae* involves the assembly of T4P. The main scope of this thesis was to study the composition of the competence pilus and for that we structurally characterized ComGC, the main building block of the pilus. Moreover, we wanted to investigate the role of the pneumococcal minor pilins in pilus biogenesis. The applicability of a label-free sorting technique to study pneumococcal subpopulation was also investigated.

2.1 Specific aims

Paper I

To characterize the major pilin ComGC as the major component of the pneumococcal competence pilus and to determine the molecular structure of this protein.

Paper II

To determine to role of the pneumococcal minor pilins ComGD, ComGE, ComGF and ComGG in competence pilus biogenesis and transformation.

Paper III

To sort and study pneumococcal subpopulations using the microfluidic label-free sorting technique named deterministic lateral displacement (DLD).

3 METHODOLOGICAL CONSIDERATIONS

In this section, a general overview of the bacterial strains and methods used in the work of this thesis will be presented. For a detailed description of the protocols, please see the Material and Methods section of the respective papers.

3.1 Growth conditions and bacterial strains

S. pneumoniae was cultured in C medium with yeast (paper I) or in Todd-Hewitt Broth with yeast extract (paper II) at 37°C. In paper III, pneumococcal strains were grown on blood agar plates incubated overnight at 37°C in 5% CO_2. In paper I and II, competence was induced by addition of competence stimulating peptide (CSP) at a final concentration of 100 ng/ml for 15 min.

Throughout the studies of this thesis, different pneumococcal strains were used: the encapsulated serotype 4, TIGR4, and its non-encapsulated isogenic derivate T4R are used in paper I. The non-encapsulated serotype 2, R6, was used in all papers and the encapsulated serotype 2, D39, was used in paper III.

In paper II, to generate pneumococcal in-frame deletion mutants, the fragment flanking the upstream and downstream regions of the target genes were amplified by PCR and ligated to a spectinomycin or erythromycin cassette. The resulting fragment was used to transform *S. pneumoniae* R6. Transformants were selected on blood agar plates containing the appropriate antibiotic and confirmed by PCR and sequencing. In addition, pneumococcal minor pilin mutants were constructed by ectopically expressing minor pilin(s) in the *bgaA* locus using integration vectors carrying pneumococcal minor pilin(s) that were transformed into *S. pneumoniae* R6. Transformants ectopically expressing minor pilin(s) in *bgaA* were selected on blood agar plates with tetracycline. Next, endogenous minor pilins were deleted accordingly by replacing the region of interest with a spectinomycin or erythromycin cassette.

In paper III, bacteria grown on blood agar plates overnight were resuspended in a PBS solution containing 1% bovine serum albumin (BSA) at $OD_{620nm} = 1$ before being sorted into different subpopulations.

3.2 Characterization of the pneumococcal competence pilus and its function

3.2.1 Transmission electron microscopy and immunogold labelling

Transmission electron microscopy and immunogold labelling was used to visualize pili on competent *S. pneumoniae* R6. In paper I, primary antibody specific to ComGC and secondary antibody conjugated to 6 nm or 10 nm gold particles were used for immunogold labelling. In paper II, the incorporation and distribution of ComGF into the filament was evaluated by using anti-ComGF antibodies and secondary goat anti-rabbit antibody conjugated to 6-nm gold particles.

3.2.2 Immunofluorescence microscopy

In paper II, competence pilus assembly in pneumococcal mutants was assessed by immunofluorescence microscopy. Competent bacteria were fixed and labelled with anti-ComGC primary antibody and Alexa 594 conjugated goat anti-rabbit secondary antibody (Life Technologies). The localization of the pneumococcal minor pilins was assessed similarly using anti-ComGD, anti-comGF, and anti-ComGG antibodies. A strain expressing an extra copy of flag tagged ComGF was used to co-stain competence pili with anti-Flag and anti-ComGC.

3.2.3 Transformation frequency

Transformation frequencies of the strains investigated in paper I and II were determined by using genomic DNA of *S. pneumoniae* carrying a streptomycin resistance mutation in the *rpsL* gene (340). Bacteria were grown until OD_{620} = 0.15 and competence was induced with CSP for 15 min at 30 °C. DNA (1 µg/ml) was then added. Bacteria were incubated 30 min at 30 °C, followed by 60 min at 37 °C and were then plated on blood plates in the presence and absence of streptomycin. The blood plates were incubated overnight at 37 °C and 5% CO_2 before colony counting.

3.2.4 Bacterial Adenylate Cyclase Two Hybrid (BACTH) system

The BACTH system allows determination of protein-protein interactions by restoring the activity of the *Bordetella pertussis* adenylate cyclase in *E. coli* reporter strains (341). In paper I and II, this approach was used to test ComGC-ComGC and ComGC-ComGG interactions, respectively. A detailed description can be found in paper I. The degree of interaction between pneumococcal pilins was measured as β-galactosidase activity, which was expressed in Miler units calculated from the following formula: 1000 x (OD_{420nm} - 1.75 x OD_{550nm})/(incubation time (minutes) x volume (1 ml) x OD_{600nm}).

3.3 Protein expression and purification

E. coli Rosetta (DE3) cells and *E. coli* T7 Express competent cells were used for heterologous expression of pneumococcal major and minor pilins, respectively. In paper I, His-tagged truncated ComGC was affinity purified, the His-tag was subsequently cleaved and untagged ComGC was further purified by size-exclusion chromatography. The sample was then used for nuclear magnetic resonance (NMR) studies and structure determination. In paper II, to test minor pilin interactions, untagged or GST-tagged soluble domains of each minor pilin were first expressed individually. Then, the pellet from a culture expressing an untagged pilin was mixed with the pellet expressing a GST-tagged pilin and each minor pilin combination was affinity purified and analysed by SDS-PAGE and Coomassie staining and immunoblotting. In addition, the minor pilin complex was also purified by affinity purification from an *E. coli* strain co-expressing all four minor pilins. The elution fraction was analysed by native polyacrylamide gel electrophoresis and Coomassie staining and detected bands were confirmed by mass spectrometry.

3.4 Co-immunoprecipitation of pneumococcal sheared pili

Co-immunoprecipitation assays were used in paper II to determine whether the pneumococcal minor pilins are associated with competence pili in *S. pneumoniae* R6. ComGC antisera was bound to Protein A Sepharose beads and incubated with sheared pili samples. Samples were analysed by SDS-PAGE and immunoblotting.

3.5 Pneumococcal cell surface hydrophobicity

The presence or absence of capsule is described to impact the hydrophobic character of the bacteria (342). In paper III, the cell surface properties of the encapsulated *S. pneumoniae* D39 and the non-encapsulated R6 were investigated by hydrocarbon adherence assay (343).

3.6 Imaging and sorting of pneumococcal subpopulations

To design devices suitable to sort R6 and D39, size measurements of these strains were performed on microscopy images taken of the cells grown overnight on blood agar plates. Devices were designed and optimized to sort *S. pneumoniae* R6 into subpopulations. To test the accuracy of this technique, sorting of the non-encapsulated R6 and the encapsulated D39 was also monitored. Time-lapses movies were taken from the samples running through the DLD device as well as movies of bacteria for each outlet. The movies from the outlets were used to blindly count the number of bacteria in each outlet according to their size allowing an unbiased characterization of each subpopulation.

4 RESULTS AND DISCUSSION

4.1 Paper I

Structure of the competence pilus major pilin ComGC in *Streptococcus pneumoniae*

S. pneumoniae is competent for natural genetic transformation, a feature that allows pneumococci to acquire new traits by taking up extracellular DNA from the surroundings and incorporating it into its genome by homologous recombination. During competence, *S. pneumoniae* R6 was shown to assemble T4P, which are composed of the major pilin ComGC (291). Thus, the first study included in this thesis biochemically characterized the major pilin ComGC in *S. pneumoniae* R6 and TIGR4 strains. We investigated direct interactions between ComGC monomers and the ability of ComGC to oligomerize into high-molecular weight structures. In addition, we also determined the NMR structure of soluble ComGC.

To visualize pneumococcal competence pili, we used the non-encapsulated T4R strain deficient in sortase-mediated pili (T4RΔ*rrgA-srtD*), to prevent the expression of other structures on the bacterial surface. Additionally, the non-encapsulated R6 strain was used, which naturally does not express sortase-mediated pili (344). After competence induction, both strains assembled filaments as visualized by transmission electron microscopy. The filaments were more abundant in competent R6, which is why this strain was used for further microscopy studies. To show that ComGC forms the backbone of the pneumococcal competence pilus, immunogold labelling was performed using antibodies specific to ComGC and secondary antibody conjugated to 6 nm or 10 nm gold particles. The electron micrographs showed that gold particles labelled the entire pilus filament, indicating that the major pilin ComGC is the main component of the pneumococcal competence pilus.

To validate that ComGC can oligomerize into pili, we further analysed pili preparations by two-dimensional PAGE. When pili samples of competent WT bacteria were run on a native gel (first dimension) and subsequently immunoblotted with ComGC antibody, a high-molecular weight band was detected. Next, a lane from the native gel was cut and placed horizontally on top of an SDS-containing polyacrylamide gel (SDS-PAGE, second dimension). During PAGE, the SDS present in the gel denatured the high-molecular weight structure formed by ComGC, and when immunoblotted with anti-ComGC, a ladder-type pattern was observed. Due to partial denaturation, high-molecular ComGC but also monomeric ComGC and distinct oligomeric forms, which correspond to ComGC dimers and trimers, were observed. This further suggests, that ComGC can oligomerize and is able to form the backbone of the competence pilus. We further detected the direct

interaction between two full-length ComGC monomers by using the bacterial adenylate cyclase two-hybrid system. Moreover, we could show *in vitro* that PilD can process full-length ComGC, when co-expressed in *E. coli.* Altogether, these results show that the major pilin ComGC has the intrinsic potential to oligomerize and assemble into T4P.

In addition to the biochemical characterization, we also studied the structural features of pneumococcal competence pili by analysing negatively stained transmission electron micrographs. We determined that the average diameter of the filaments was 64 ± 1.6 Å, which is comparable to the diameter of T4P in *N. gonorrhoeae* (60 Å) but much larger than T4P in *T. thermophilus,* with a diameter of approximately 30 Å (132, 191). The differences observed are likely the result of structural differences of the major pilins, which can differ substantially in sequence and size. The observed competence pili also exhibited a high degree of flexibility and after analysing a large number of micrographs, a suitable number of straight regions in the filaments was identified and used to examine the potential helical symmetry in the filaments. Based on a class average power spectrum, it was possible to determine that the helical pitch of the pilus was approximately 40 Å. This suggests that pneumococcal T4P are somewhat less compact, when compared to the helical pitch of 37 Å of *N. gonorrhoeae* T4P. In contrast, pneumococcal pili are more compact than T4P in *T. thermophilus*, which have a helical pitch of 49 Å (132, 191).

The NMR structure of soluble ComGC solved in this study has shown distinct structural features. In Gram-negative bacteria, type IV pilins often present a "lollipop-type" of conformation. However, ComGC is exclusively formed by α-helices (α1-C, α2 and α3) with a small head domain. Another important observation was the lack of cysteines in ComGC. Most type IV pilins have two cysteine residues in their C-terminal, which are involved in pilin stability and polymerization (107). This raises the question of how the pneumococcal competence pilus is stabilized. The lack of cysteines is not exclusive for *S. pneumoniae*, as the major pilin (PilA1) in *C. difficile* also lacks them. However, the structure of PilA1 differs from ComGC and it presents a more compact C-terminus stabilized by two β strands, which suggests distinct pilus stabilization strategies used by *C. difficile* and *S. pneumoniae* (345). The lack of structural stabilizing elements and the presence of a small head domain solely formed by α2 and α3 may explain the high level of ComGC flexibility observed. The highly dynamic properties of ComGC in solution may be an important characteristic for pilus assembly and function.

When analysing the electrostatic potential of the solvent-accessible surface of ComGC, a well-defined electropositive cavity was identified along the N-terminal region of ComGC. The electropositive cavity contains exposed lysine and arginine

residues, which may contribute to DNA binding as observed for other DNA-binding proteins (346). This is an interesting observation that may help to understand how DNA is bound by competence pili. Interestingly, there was a recent attempt to model monomeric ComGC into a filament (175). The model hypothesized that the latter portion of the α1-N helix and the α1-C helix form a continuous α1, which form the core of the filament. The head domain formed by α2 and α3 is highly flexible, but by estimating the geometric centre (centroid) of the residues from α2 and α3, Piepenbrink suggested a pilus model with a diameter of approximately 60 Å, somewhat similar to our observations (175). Interestingly, when comparing the structure of monomeric ComGC with this model, the electropositive patch would be surface-exposed, being consistent with our suggestion that this region has potential DNA binding properties.

In summary, the work presented in this paper represents the first structure of a major pilin protein from a competence system and provided initial structural insights regarding competence pilus assembly in *S. pneumoniae*.

4.2 Paper II

Pneumococcal competence pilus assembly requires formation of a minor pilin complex

In the second study of this thesis, we further investigated the assembly of competence pili in *S. pneumoniae* with particular focus on the pneumococcal minor pilins. In addition to ComGC, four minor pilins (ComGD, ComGE, ComGF and ComGG) are also encoded by the *comG* operon and expressed by competent pneumococci. Thus, we aimed at understanding the role of the pneumococcal minor pilins in competence pilus assembly and function.

It is important to note that the genes of the *comG* operon are overlapping, making the deletion of individual genes for further functional studies complicated. Consequently, we constructed in-frame deletion mutants lacking one or more minor pilins *en bloc* and evaluated their ability to assemble competence pili. This was assessed by immunoblotting and by analysing the levels of ComGC present in cell lysates and supernatants of the constructed minor pilin mutants. In general, lower levels of the major pilin ComGC were detected in all mutants but ComGC processing was not visibly affected. In addition, no ComGC was detected in the supernatant of these strains suggesting that pilus assembly was compromised. Since the competence pilus is essential for transformation, we also determined the transformation frequency of each minor pilin mutant and we confirmed that they were non-transformable compared to the WT strain. These results indicate that the minor pilins are important for pilus assembly and function. Similar observations were reported in *B. subtilis,* which expresses a homologous *comG* operon (347, 348).

Next, we tested whether pilus assembly and function could be restored in a mutant lacking all four minor pilins by ectopically expressing individual pilins or pilin combinations in the *bgaA* locus. We observed that pilus assembly and transformation were only restored in the complemented strain expressing all four minor pilins. Interestingly, in the complementation strains expressing single *comGG* or *comGDEFG* the levels of ComGC in the cell lysates were similar to the WT strain suggesting that ComGG alone is able to stabilize ComGC. However, ComGG alone is not sufficient to restore pilus assembly and transformation.

Since ComGG was able to stabilize ComGC in absence of other minor pilins, we hypothesized that the two proteins would interact directly. By using the BACTH system implemented in paper I, we confirmed that full-length ComGC and ComGG interact directly. Considering that the presence of all minor pilins is required to assemble functional pili, we investigated whether there is a possible link between ComGG and other minor pilins. By immunoblotting, we observed that the stability

of ComGD and ComGF were strongly affected in a R6Δ*comGG* strain. The stability of ComGE could not be assessed at this stage as the respective antisera failed to detect the protein in pneumococcal lysates. Altogether, these results indicate that ComGG also interacts with ComGD and ComGF and it is crucial for their stability.

Interactions between minor pilins have been reported in other systems (349, 350) and our data suggest that ComGG is likely to have a key role in the interaction with other pneumococcal minor pilins. This led us to evaluate the protein-protein interaction network among pneumococcal pilins in *E. coli.* For that, the soluble domains of each pilin, GST-tagged or untagged, were expressed independently. Next, we tested different binary pilin combinations by mixing and affinity purifying the pellets of untagged pilins with the pellets of each GST-tagged pilin. Our results indicate that the soluble domains of ComGD, ComGE, ComGF and ComGG can interact with each other. However, no interactions were detected in the affinity purification experiments using soluble GST-tagged ComGC. These results are in accordance with observations reported in other systems including *B. subtilis* and *P. aeruginosa* (349, 350), and also suggest that pneumococcal minor pilins are likely to form a minor pilin complex. To validate this hypothesis, we co-expressed all minor pilins in *E. coli,* which were then affinity purified. The eluate fraction was loaded onto a native gel and after Coomassie staining, a distinct band corresponding to approximately 66 kDa was visible. Mass-spectrometry analysis confirmed that this band corresponded to a complex formed by the pneumococcal minor pilins. The formation of a minor pilin complex has also been reported in other systems like *B. subtilis* and *P. aeruginosa* (349, 350).

Next, we investigated the presence of minor pilins in cell lysates and sheared pili fractions in competent R6. We observed that ComGD, ComGF and ComGG can be found both in cell lysates and in sheared pili of competent R6. All three minor pilins were also detected after co-immunoprecipitation of sheared pili using anti-ComGC antibodies. In addition, by immunofluorescence and electron microscopy analysis we clearly visualized that ComGF is integrated throughout the competence pilus. Similarly, minor pilins in *P. aeruginosa* and *Clostridium difficile* were shown to be incorporated throughout T4P (345, 351). However, we were unable to detect ComGD and ComGG in the filaments assembled by competent R6, despite repeated attempts. This can be a result of the low abundance of these proteins in the filament or due to limited accessibility of the epitopes recognized by the antibodies. Considering our previous results that ComGG stabilizes ComGF and that minor pilins form a complex, we cannot rule out the presence of other minor pilins or the entire minor pilin complex in the filament.

The presence of the invariant E5 residue is a conserved feature among type IV pilins and it has been shown that a point mutation of E5 in ComGC (E5V) abolished

competence pilus assembly and transformation (291). Since all pneumococcal minor pilins, except ComGG, present the invariant E5 residue we tested the role of the E5 residue present in ComGD, ComGE and ComGF in pilus assembly and transformation. All mutants carrying the E5A mutation presented similar amounts of ComGC in the cell lysates compared to the R6 strain suggesting that pilin processing is not affected. However, the levels of ComGC in sheared pili fractions showed variation among mutants. Competent pneumococci expressing $ComGD_{E5A}$ or $ComGE_{E5A}$ variants showed reduced levels of ComGC whereas bacteria expressing $ComGF_{E5A}$ presented only slightly decreased amounts of ComGC compared to the R6 strain. When the transformation frequency of those strains was determined, we observed a correlation with the amount of ComGC detected in the sheared pili and the transformability of each mutant. The transformation frequency was significantly lower in $ComGD_{E5A}$ and $ComGE_{E5A}$ mutants and no difference was observed in the $ComGF_{E5A}$ variant. Together, our results indicate that the E5 residue present in ComGD and ComGE is important for competence pilus biogenesis and function.

Pneumococcal ComGG lacks the E5 residue and is larger than the major pilin ComGC. Similarly, other systems including the *P. aeruginosa* T4P and *Klebsiella* T2SS also present a large minor pilin lacking the E5 residue which is thought to be part of the pilus tip (352, 353). While we are lacking evidence for the presence of ComGG in the tip of the competence pilus, our data strongly suggest that ComGG is a key link between the major pilin and the minor pilin complex.

In summary, our results support the formation of a minor pilin complex to prime competence pilus formation and demonstrate a key role for ComGG as the linker between the major pilin and the minor pilin complex.

4.3 Paper III

Separation of pathogenic bacteria by chain length

S. pneumoniae can grow in different cell arrangements, such as single cocci, diplococci or chains of variable lengths. Pneumococcal cell size has been associated with the ability to colonize or cause disease. Chain formation promotes adhesion to epithelial cells *in vitro* and *in vivo* while short chains and single cocci have been associated with immune response evasion and meningitis in a mouse model (231, 232). Chaining mutants were also described to have a longer competence window *in vitro* when exposed to antibiotics, such as aztreonam and clavulanic acid (284). Competence induction has been linked to promote biofilm formation, which is associated with colonization *in vivo*, as well as to enhance virulence in mouse models of pneumonia (312, 316). Thus, the separation of pneumococci into subpopulations would enable to study the relation between competence and bacterial cell size in pneumococcal pathogenesis. Until now, label-free sorting techniques like DLD have not been previously used to sort *S. pneumoniae* into distinct subpopulations. The third study in this thesis aimed therefore at testing the applicability of DLD to label-free sorting of *S. pneumoniae* subpopulations.

The DLD devices used in this study were designed to sort the non-encapsulated *S. pneumoniae* R6 strain and the closely related encapsulated D39 strain into distinct subpopulations based on cell size and morphology. The devices were 9.5 mm long with two inlets (one to load the bacterial sample and another to add the buffer) and five outlets, each one designed to collect a different bacterial fraction which was displaced differently in the device. The critical diameter of the device was 1.24 µm, which is larger than the average size of single cocci (≈1 µm), smaller than the size of diplococci and chains, but larger than their length. Based on a previous study in which DLD was used to sort trypanosomes, the depth of the device was chosen to be approximately 10 µm (337). Both trypanosomes and pneumococcal chains can be considered as long thin biological particles; a depth of 10 µm is expected to increase the effective size of the chains allowing proper displacement of longer chains in relation to other morphologies (shorter chains, diplococci and single cocci). To prevent clogging of the device, a solution of poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) was used to coat the devices and to create a hydrophilic surface that minimizes bacterial adhesion to the surface and posts inside the device.

Bacterial solutions were prepared from pneumococci grown overnight on blood agar plates, resuspended in PBS containing 1% (w/v) BSA and adjusted to a final optical density (OD_{620nm}) of 1. Before sorting, we characterized the input bacterial suspensions by measuring the cell size distribution of D39 (n=226) and R6 (n=146) cells. Overall, we observed that the encapsulated D39 strain presented in average

wider (1.09 mm vs 0.92 mm) and longer (1.43 mm vs 1.32 mm) cocci compared to the non-encapsulated R6, which could be related to the presence of capsule in the D39 strain. The D39 strain also formed longer chains compared to the R6 strain (4.82 µm vs 3.78 µm). Remarkably, when we grouped the measurements for each strain into the different cell arrangements (single cocci, diplococci, and chains), we observed a substantial overlap in sizes between the different cell types. It is worth to note that in these experiments, we used bacteria grown overnight on blood agar plates. This is likely to result in bacteria in different stages in their replication cycle or in distinct metabolic stages depending on nutrient accessibility. It would be interesting to test synchronized cultures to determine whether these differences will still be observable. Next, we determined the proportion of each cell type in each unsorted sample. Both strains are mainly composed of diplococci, ≈80% in the R6 strain and ≈60% in the D39 strain. Single cocci and chains were similarly distributed in the unsorted R6 strain while in the unsorted D39 strain, single cocci accounted for ≈30% of the sample and chains ≈10%.

Bacterial suspensions were sorted individually during 2.5 h during which movies from the outlets were taken to determine the ratio of single cocci, diplococci, and chains in each outlet reservoir. Sorting was run until the concentration of pneumococci in the outlets was sufficiently high to count tens to hundreds of bacteria unequivocally. At least 4 movies in distinct areas were taken per outlet. Then, the movies were randomized, analysed in a blind fashion to avoid bias in the characterization of each fraction, and the cell types were counted manually. Overall, we observed equivalent separation patterns in both strains. Single cocci were mostly found in outlets 1-3, which correspond to outlets in which bacteria were not, or little, displaced in the device. Chains were exclusively found in the outlets 4 and 5 and represent the fractions, which were displaced the most during sorting. Diplococci were found in all outlets, with particular enrichment in the outlets collecting bacteria, which were displaced. Considering our previous measurements in which the length range of diplococci vastly overlapped with the length of chains (and to a smaller extent with single cocci), this result is not totally unexpected. This reflects the sensitivity of DLD to distinguish small variations in size. A possible way to circumvent this would be by increasing the length and the width of the array which would allow bacteria, especially diplococci and chains, to be sorted more efficiently. Alternatively, adjustments in the size of the gaps in the array could allow us to improve the purity of single cocci or chains.

Since the polysaccharide capsule represents a major virulence factor of *S. pneumoniae*, we sought to investigate whether we could separate the encapsulated strain D39 from the non-encapsulated strain R6. In order to distinguish the two strains following sorting, we used a GFP-labelled D39 strain. After sorting, we clearly observed an enrichment of fluorescent bacteria in the outlets 4 and 5.

The non-fluorescent R6 strain was found enriched and equally distributed in the outlets 1-3. As larger cells are expected to be displaced and to be collected primarily in outlets 4 and 5, these observations are in line with our measurements showing that the D39 strain has overall larger cell sizes. It has been reported that the encapsulated D39 strain and a non-encapsulated variant presented distinct hydrophobic profiles in which the non-encapsulated strain was substantially more hydrophobic, estimated by ability to adhere to hexadecane (343). In accordance with these results, we observed that the non-encapsulated R6 strain displayed a higher hydrophobic character (42.4 ± 2.10% adherence to hexadecane) compared to the D39 strain (2.7 ± 0.16% adherence to hexadecane). This may be an indication that D39 and R6 strains interact differently with the surface of the device. However, it remains unknown if the different hydrophobicity characters contributed to the sorting efficiency.

During our experiments, we also followed the trajectories of pneumococci in the devices and observed that bacteria ran through them in several modes, which reflected in trajectories ranging from zero to maximum displacement. Larger particles, like chains, experienced rotation and bending due to their flexibility. While running in the device, chains moved in different modes, which can be triggered by different factors including variations in the particle shape and bending properties or fluctuations in the fluid flow. These different transport modes influence the effective size of bacteria and will lead to displacement or not. However, wider and longer particles like chains are more likely to move in modes that cause an increasing displacement and subsequently being sorted from smaller particles. Other factors including differences in deformability may also influence sorting, however, in this study, we did not evaluate if D39 and R6 strains were differently deformable. In summary, while hard spherical particles migrate only in two modes (zig-zag mode or displacement mode), the size, shape and orientation of non-spherical biological particles like pneumococci preparations can vary leading to particles migrating differently depending on their effective size.

Sample recovery is a major challenge in many microfluidics devices including DLD (333). Importantly, we succeeded in recovering sorted samples from each outlet, albeit in low concentrations, encouraging further optimizations that would increase sample recovery. The throughput of the device was estimated to be 1 µl/h which can be optimized by using wider devices, larger spacing between posts, parallelization and fine-tuning the flow velocity. However, this needs to take in consideration for the sample concentration and duration of sorting to prevent clogging of the device.

Finally, and equally important, we obtained a bacterial viability for each strain higher than 87% after running the sorting experiment for 2.5 hours. This is a crucial aspect to consider when thinking in downstream analysis of the subpopulations.

To summarize, in this work we present solid evidence that DLD is a suitable technique to sort *S. pneumoniae* into different subpopulations. In addition, we show that the sensitivity of this technique allows separation of mixed pneumococcal cultures based on small morphological differences as the presence or absence of capsule. Thus, DLD also has the potential to be applied to sort pneumococcal isolates which often lack capsule and are serologically non-typeable (354, 355) into subpopulations to gain a deeper detail in the dynamics of colonization, a crucial requirement for invasive disease.

5 CONCLUDING REMARKS

Horizontal gene transfer plays a key role in the dynamics of bacterial genomes, and natural competence for transformation in *S. pneumoniae* vastly contributes to its genome plasticity. During competence, pneumococci assemble T4P, which are essential for natural transformation, making them crucial structures involved in the DNA uptake. Through natural transformation, *S. pneumoniae* can acquire antibiotic resistance genes and other advantageous traits that can improve its fitness and ability to colonize and cause disease. Consequently, a comprehensive understanding of the competence pilus biogenesis is required.

In paper I, we gained a first structural understanding of the major component of the competence pilus. The NMR structure of ComGC revealed distinct features that differ from canonical type IV pilins. The assembly of long and flexible T4P may help pneumococci to capture DNA at long distances from the bacterial surface. This could provide an advantage to minimize repulsive electrostatic interactions between the transforming DNA and bacterial surface. Moreover, long and flexible competence pili may facilitate better binding of DNA, leading to enhanced DNA uptake of that strain. Finally, identification of regions with potential DNA binding is an important feature to further investigate. A cryo-electron microscopy structure of the competence pilus and modeling of the individual subunits into this model would provide more information on how DNA is bound by the pilus. Interestingly, the high-resolution crystal structure of gonococcal PilE and 3D cryo-electron microscopy reconstruction of the pilus filament led to the identification of positively charged grooves suitable to wrap DNA (191).

It is commonly believed that DNA uptake occurs at the pneumococcal mid cell coinciding with the site of peptidoglycan synthesis, which was proposed to facilitate DNA uptake through the thick peptidoglycan layer (294). The extension of the pilus through the cell wall still remains a puzzling question. Moreover, there is currently no evidence that the pneumococcal competence pilus retracts. In contrast to other T4P systems, the pneumococcal competence pilus system lacks a clear retraction ATPase. Interestingly, in *C. crescentus,* it was shown that tad-T4P, which lack a retraction ATPase, retract due to the action of a bifunctional ATPase that powers both pilus extension and retraction (128). Whether, pneumococcal ComGA could function as bifunctional ATPase that mediates both pilus extension and retraction, remains to be investigated. Alternatively, it has been shown that a minor pilin, most likely located at the tip of the pilus, promotes the retraction of TCP in *V. cholerae* (129).

In paper II, we show that pneumococcal minor pilins are essential for natural transformation and unveiled a complex interaction network. We visualized that

ComGF is incorporated throughout the filaments, however; we cannot rule out that other minor pilins or the entire minor pilin complex can be part of the pilus. The atypical features of the minor pilin ComGG and its role as the linker between the major pilin and the minor pilin complex are also compatible with the idea that the minor pilin complex could be located at the tip of the pilus, possibly to promote the binding of DNA and/or pilus retraction. Clearly, more experimental evidence is needed to investigate this possibility.

While the competence pilus is not required for virulence in *S. pneumoniae* D39, which under laboratory conditions has a low transformability rate, deletion of the major pilin *comGC* in hyper-transformable pneumococcal isolates affected colonization and virulence *in vivo* (309, 356). Thus, deciphering the molecular details of how DNA is taken up by pneumococci will help to identify possible innovative approaches to tackle pneumococcal infections. Compounds active against T4P-related functions, such as twitching motility and bacterial aggregation, have being identified in pathogenic *Neisseria* species and *P. aeruginosa* (357). In *N. meningitidis*, these compounds interfered with the sodium gradient, and possibly with the proton motive force, through the inner membrane and promoted pilus retraction over pilus extension through a yet unknown mechanism (357). Interestingly, pneumococcal competence inhibitors disrupting the proton motive force and CSP export have also been recently identified (319).

Moreover, the pneumococcal competence regulon also plays an important role in regulating pneumococcal colonization and virulence. There is also evidence that the size of pneumococci is important in the interplay between colonization and invasive disease. Thus, separating *S. pneumoniae* into distinct subpopulations based on their size could be an interesting approach to study how competence is regulated at subpopulation level and how this would impact colonization and virulence. In paper III, we validated the applicability of microfluidics to study *S. pneumoniae* and believe that this approach has great potential to further study pneumococcal colonization and invasive disease properties at subpopulation level.

In conclusion, the work included in this thesis has laid a basic foundation to better understand the importance of pneumococcal competence pili, not only in DNA uptake but also in the pathogenesis of this bacterium. Moreover, we identified DLD as an attractive technique, which offers the possibility to further study the link between competence, colonization, and virulence based on the morphological properties of pneumococci.

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